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135

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CONTENTS

Potassium Deficiency and Excess in Guayule. II. Cation-Anion Balance in the Leaves. Bruce J. Cooil, Hawaii Agricultural Experiment Station, Honolulu, Hawaii 403

Effects of Potassium Deficiency and Excess Upon Certain Carbohydrate and Nitrogenous Constituents in Guayule. Bruce J. Cooil, and M. G. Slattery, Hawaii Agricultural Experiment Station, Honolulu, Hawaii 425

Investigations on the Fluorometric Determination of Malic and Succinic Acids in Apple Tissue. C. Guinn Barr, Michigan Agricultural Experiment Station, East Lansing, Michigan 443

The Absorption and Fluorescence Spectra of Two Red Marine Algae. R. W. Van Norman, C. S. French, and Fergus D. H. MacDowall, University of Minnesota, Minneapolis 455

Physiological Studies of Weed Seed Germination. Robert O. Bibbey, Science Service, Department of Agriculture, Ottawa, Canada 467

Rate of Leaf Elongation as Affected by the Intensity of the Total Soil Moisture Stress. C. H. Wadleigh and H. G. Gauch, U. S. Regional Salinity and Rubidoux Laboratories, Riverside, California, and Agricultural Research Administration, U. S. Department of Agriculture 485

Gaseous Losses of Nitrogen from Green Plants. I. Studies with Chlorella and Lemna. F. E. Allison, Katharine S. Love, L. A. Pugsk and V. L. Gaddy, Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture, Beltsville, Maryland 496

(*Continued on Cover Page Four*)

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PLANT PHYSIOLOGY

OCTOBER, 1948

POTASSIUM DEFICIENCY AND EXCESS IN GUAYULE. II. CATION–ANION BALANCE IN THE LEAVES

BRUCE J. COOIL

(WITH ONE FIGURE)

Received June 2, 1947

In many plants the potassium contained in the tissues is almost completely water soluble (**3, 15, 16, 18, 19, 32**), although this is not invariably the case (**8**). Potassium is frequently the chief soluble mineral cation present in the cellular fluids, particularly in plants which normally contain little calcium, such as wheat and bluegrass, and in those in which most of the calcium is present in insoluble forms such as spinach and buckwheat (**8, 21**). As emphasized by HOAGLAND (**13**) with particular reference to plants deficient in potassium a decrease in fixed base in the sap may result if much of the calcium and magnesium goes out of solution in the plant, even though the content of total calcium and magnesium in the tissues as a whole increases by amounts equivalent to the decreased absorption of potassium.

It is generally supposed that much of the potassium present in plants is in the form of salts of organic acids and that these acids and their salts constitute principal components of the buffer systems. A relationship between the total organic acids and the excess of cations over inorganic anions in tobacco leaves of different ages and base composition was shown by PUCHER, VICKERY, and WAKEMAN (**22**). PIERCE and APPLEMAN (**21**) grew several species of plants in the same nutrient medium and showed a general correlation between the excess of cations and the total organic acids. In these correlations, however, were included both soluble and insoluble constituents, and variations in insoluble oxalate and calcium contributed much to the variations in the total bases and organic acids. ULRICH (**33**) has shown that the total organic acid content of sap expressed from barley roots may be varied by unequal absorption of cations and anions from the solution. An absorption of cations in excess of anions results in an in-

[1] Contribution from the Guayule Research Project, Bureau of Plant Industry, Soils and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture. The cooperation of the U. S. Forest Service is gratefully acknowledged. Clerical assistance was provided by the U. S. Regional Salinity Laboratory.

crease in organic acids. By employing calcium bromide, the organic acid content of the roots can be shown to decrease when bromide ions are absorbed in excess of calcium ions.

Effects of different rates of potassium supply upon the growth and mineral contents of guayule plants have been reported in a previous publication (5). It was noted that although the nutrient treatments resulted in large differences in concentrations of potassium and other cations within the plants, the total milliequivalents of cations per 100 grams of dry tissue for the whole plants was nearly the same for all treatments. In this paper are reported results from a study of the cation-anion balance in the leaves of the guayule plants previously described (5). An attempt was made to determine the effects of variations in contents of potassium and concomitant variations in contents of other cations upon the distribution of ions between soluble and insoluble fractions.

Materials and methods

The culture methods employed in growing the experimental plants have been described (5). Treatments designated K_1, K_2, and K_3 comprised series 1. The nutrient solutions for these treatments contained 0.5, 3, and 12 milliequivalents K per liter, respectively. These solutions each contained 10 m.e. Ca per liter and no Na except as impurities. Treatments designated NaK_1 NaK_2, and NaK_3 comprised series 2. The solutions supplied to these plants contained the same concentrations of K as the corresponding solutions of series 1, but each solution of series 2 contained 4 m.e. Ca and 6 m.e. Na per liter.

At final harvest 100 grams of fresh tissue from each leaf sample were tightly sealed in one quart fruit jars. The jars were packed in dry ice and stored there until immediately preceding analysis (at least 48 hours). Rapid and carefully controlled thawing was found to be essential to obtain reproducible estimations of the carbonate components. To this end, the frozen sample was rapidly transferred to a two quart jar. The jar was then sealed and rotated in water at 50° C. for 15 minutes. At this point the material was completely thawed but still cold. The sample was then placed in a muslin cloth and subjected to 1300 pounds pressure per square inch (effective pressure at the surface of the tissue mass) in a Carver hydraulic press. This pressure was maintained until flow of the sap had practically ceased. The pressing operation required six to seven minutes.

For total solids, five ml. samples of expressed sap were evaporated to about 1 ml. on a water bath and dried to constant weight in a vacuum oven at 65° C. and 3 to 4 cm. pressure. For specific gravity, five-ml. aliquots of fresh sap were weighed in weighing bottles of 5 ml. capacity. The sample used for determining total solids was ashed by the magnesium nitrate method (1). Sulphur in the ash solution was determined by the official method (1). A separate aliquot was employed for the determination of phosphorus by the method of Fiske and Subbarow (9).

For calcium and magnesium, five ml. aliquots of sap were taken to dryness on a steam bath, moistened with sulphuric acid (1–10), dried in an oven at 105° C. and ashed at 600° C. for 16 hours. The ash was dissolved in dilute HCl, taken to dryness to dehydrate any silica, redissolved in HCl. Calcium and magnesium in the ash solutions were determined by the official method (**1**). Potassium was determined by the method of HIBBARD and STOUT (**12**). Nitrates were determined by the phenoldisulfonic method (**10**) as described by SCHLENKER (**28**).

For total non-volatile organic acids, malic acid, and citric acid, 10 ml. aliquots were concentrated to 1–2 ml. in a vacuum oven at 50° C. and 3–4 cm. pressure. The ether extraction was performed in a Goldfisch extraction apparatus. Twelve-hour extraction periods were employed. A Beckman pH meter was used for titration of the ether extracts. The procedures were otherwise identical to those described by PUCHER, VICKERY and WAKEMAN (**23, 24**), and PUCHER, VICKERY and LEAVENWORTH (**25**).

Bicarbonate was estimated from the carbon dioxide which could be aspirated from the sap after acidification with hydrochloric acid containing stannous chloride. The procedure used was essentially the official method for determination of carbonate in soils (**1**). In place of the sample flask, a 175 mm. × 22 mm. test tube was used. A stream of carbon dioxide-free air was introduced at the bottom of the test tube through a Folin bulb. The samples were aspirated slowly for two hours into the absorption towers. Five ml. of sap were introduced into the test tube immediately after expression; 1.3 ml. of 33 per cent. $SnCl_2$ in HCl (1–1) were added rapidly and aspiration begun. Blank determinations were run with each series. Quantitative recovery of carbon dioxide was obtained from standard solutions of sodium carbonate.

For the analysis of press cakes, they were weighed immediately after expression of the sap, then dried to constant weight in a vacuum oven at 65° C. and 3–4 cm. pressure. The dried press cakes were ground to a fine powder in a micro Wiley mill. One-half gram samples were used for the determination of calcium, magnesium, sulphur, and phosphorus. The procedures were the same as those employed for the dried sap. Total non-volatile organic acids in the press cakes were determind as in the sap. 2.00 gram portions were used for this purpose. Individual acids were not determined in view of the relatively low results obtained from all samples. The phosphorus contents of the ether extracts were determined and corrections applied as described by PUCHER, VICKERY and LEAVENWORTH (**25**). Carbonate in the press cakes was determined essentially as was the bicarbonate content of the sap. Eight ml. of 10 per cent. $SnCl_2$ dissolved in HCl (1–9) were used for acidification of 0.5-gram samples. During the aspiration the tubes were shaken until the sample was thoroughly wetted.

Sodium was determined in the dried leaves samples (**5**). In some cases, dried samples were also employed for determination of potassium.

Calculation and expression of results

Estimation of sap soluble constituents was based on the work of Sayre and Morris (**26, 27**) for estimates of total constituents (i.e., soluble + insoluble). The contributions from the sap and from the press cake were calculated from the proportions of the total fresh weight represented by sap and press cake respectively. Comparable results could be obtained from dried whole leaf samples as is shown by a comparison of potassium contents of the whole leaves based on determination of potassium in the sap and press cakes, or in whole leaf samples (table I).

TABLE I

Potassium content of mature leaf tissue based on compositions of sap and press cake and on composition of dried whole leaf samples

Treatment	Milliequivalents per kilogram of fresh tissue			
	Total K			Soluble K
	Sap plus press cake	Whole dried sample	Average	
K_1	73	71	72	65
K_2	247	243	245	233
K_3	429	427	428	423
NaK_1	119	125	122	116
NaK_2	289	287	288	288
NaK_3	447	457	452	448
Average	267.0	268.3	.....	262.2

A comparison of soluble and total potassium is also shown in table I. A very small insoluble fraction is indicated, in agreement with results recorded for tissues of other plants (**19, 20**). However, in this case the insoluble fraction approaches so nearly the magnitude of the accuracy with which the determinations could be made, that a separate account of it does not seem appropriate. Accordingly potassium has been expressed as entirely soluble. The relatively small quantities of sodium found in the leaves have also been considered as soluble.

Samples from each of the eighteen plots (i.e., 3 replicates of each treatment) were analyzed separately. Analysis of variance (**30**) was applied to the analytical data.

Results

Recovery of carbonate from the leaves

It was evident from preliminary observations that quantities of the components of the carbonic acid system were present in guayule leaves and that this system would require consideration in the cation-anion balance of the leaves. Small (**29**) has pointed out that in all experiments with expressed sap the escape of carbon dioxide will be a source of error, especially where a bicarbonate system is present, as the bicarbonate-carbonic

acid ratio in the uninjured cell may differ from that which will result when the carbon dioxide content forms an equilibrium with the air. In the present study the primary object was to obtain sufficiently complete recovery of the various cations and anions present. A second consideration was the fractionation of soluble and insoluble components. The question was, whether by analysis of the expressed sap and of the press cakes a satisfactory separation of soluble and insoluble components of the carbonate system could be obtained without too great an error resulting from loss of carbon dioxide. Accordingly experiments were devised to determine the recovery which could be obtained by various procedures.

A 500-gram sample of young (expanding) leaves was gathered from 24

TABLE II

EFFECTS OF PRETREATMENT UPON RECOVERY OF CARBONATE AND UPON DISTRIBUTION OF CARBONATE AND PHOSPHATE BETWEEN SOLUBLE AND INSOLUBLE FRACTIONS

DESIGNATION AND PRETREATMENT	MILLIMOLES PER KILOGRAM OF FRESH LEAF TISSUE						pH OF SAP OR EXTRACT
	CARBONATE			PHOSPHATE			
	SAP SOLUBLE	INSOLUBLE	TOTAL	SAP SOLUBLE	INSOLUBLE	TOTAL	
A_1. Frozen in "dry ice," thawed 15 min. at 50° C.	14 ± 0.9	218 ± 5.4	232 ± 5.2	9.2	20.6	29.8	6.66
A_2. Frozen in "dry ice," thawed 2 hrs. at room temperature	38 ± 3.8	183 ± 0.3	221 ± 4.2	4.4	26.5	30.9	7.10
A_3. Frozen in "dry ice," thawed 7 hrs. at room temperature	58 ± 3.5	165 ± 5.6	223 ± 5.5	1.5	28.3	29.8	7.42
B. Frozen in brine at ca. −20° C.; thawed 5 min. at 50° C.	5 ± 0.7	231 ± 5.2	236 ± 5.8	7.4	24.6	32.0	6.57
WF. Fresh leaf tissue	.....	.....	249 ± 3.0	.....	.....	.....	7.77–8.20

plants which had received a complete nutrient solution for four months. The sample was thoroughly mixed and triplicate aliquots were employed for each of the treatments indicated in table II. The samples to be frozen were packed loosely in pint fruit jars and tightly sealed. After thawing the tissue and expressing the sap, the pH was determined and 5-ml. portions were taken immediately for determination of carbonic acid.

A 4-ml. aliquot from each sample was combined with other replicates receiving the same treatment. This mixture was used for determination of specific gravity and phosphorus content. The press cakes were handled in the same manner as in the main experiment. The ground fresh samples (WF) were rinsed into the reaction tubes with freshly boiled and cooled distilled water and made to a final volume of about 20 ml. Four ml. of

HCl (1–1) containing 33 per cent. $SnCl_2$ were added for liberation of carbon dioxide.

Results from determinations of pH, total carbonate and phosphate as well as distribution between soluble and insoluble fractions are shown in table II. Carbonate and phosphate components are expressed as millimoles per kilogram of fresh material. Recovery of total carbonate was highest from unfrozen samples, where fractionation was not attempted (WF). It is evident that the concentration of carbonate components in the sap increased with increasing time of thawing. These increases coincided with decreases in the insoluble fraction. Components of the phosphate system were much lower in concentration than carbonate components, but a decreasing concentration of phosphorus in the sap coincided with increasing amounts of insoluble phosphorus when thawing time was increased. The pH of the sap increased as the time of thawing was prolonged.

Where fresh leaf samples were ground with distilled water, pH values of the dilute suspensions were higher than any of those found in the expressed sap. The relatively high pH values of these suspensions may be explained as an effect of dilution. From results to be presented later (tables IV, V, and VII) it is evident that carbonate was the principal insoluble anion present in the leaves. Dilution of the tissue fluids, therefore, would result in solution of increased quantities of carbonate, but would not appreciably alter the quantities of other anions in solution. Thus, uncombined acids originally present in the tissue fluids would tend to be neutralized. At pH 7.8 at least 96 per cent. of any carbonic acid in solution would be in the form of bicarbonate. It is not likely that any detectable loss of carbon dioxide would be encountered, within the short time required for grinding the tissue and transferring it to the reaction flask. In fact, in these preparations it seems likely that most of the carbon dioxide which was present in the free form in the fresh tissue was recovered. To this extent, this procedure may yield high estimates of the ionic forms of the carbonate system.

In a search for the locus of the insoluble carbonate within the leaf, fresh transverse sections were examined but no inclusions of carbonate could be found in the chlorenchymous tissues. However, strips of epidermis mounted in water gave rise to a considerable evolution of gas when treated with dilute acid. Bubbles could be seen coming from within the epidermal trichomes. No such evolution could be observed from the chlorenchymatous cells when transverse sections were similarly treated with dilute acid. In interpreting the results reported in table II, it must be considered that the insoluble carbonate is localized largely in the epidermal trichomes. When the protoplasm of the leaf cells is disorganized by freezing and thawing, the insoluble carbonate is exposed to an admixture of the tissue fluids. Solution and neutralization of carbonate takes place, and precipitation of phosphate results as the pH of the sap is increased. Although these effects cannot be eliminated completely where the whole leaves are extracted or

pressed, they are minimized by rapid thawing and pressing. Recovery of total carbonate was not impaired seriously where thawing was rapid. The freezing procedure avoids dilution effects attending extraction of the fresh leaves by grinding.

Results from the main experiment

In the work of Pucher, Vickery and Wakeman (**22**), as well as in that of Pierce and Appleman (**21**), approximations have been employed in estimating some of the constituents. Thus the calcium, magnesium, sulphur and phosphorus are certainly not entirely in the ionic forms indicated. For instance portions of the insoluble calcium must be presumed to be in the form of pectates and other organic combinations not taken into account. Likewise some of the insoluble magnesium is certainly within the chlorophyll molecule. A portion of the sulphur is incorporated in the protein and amino acid molecules. In the tobacco leaf tissues employed by Pucher and Vickery, approximately 70 per cent. of the total sulphur was found to be in the form of sulphate, whereas Pierce and Appleman expressed their total sulphur values as sulphate. In the present study, the latter procedure is adopted with respect to sulphur, as no estimate of the proportion of organic sulphur is available for guayule leaves. In the previous studies (*loc. cit.*) total phosphorus has been expressed as dihydrogen phosphate, as was justified by the reactions of the tissues employed, with the assumption that the phosphorus in organic forms such as phosphoproteins, phospholipids, glycophosphates, etc. is quantitatively negligible.

In guayule leaves, the relatively high pH values and the presence of the carbonate system introduce additional errors, particularly involving the fractionation into soluble and insoluble components as noted above. Although an attempt was made to minimize these errors by employing a minimum uniform time for thawing and pressing operations, it seems improbable that soluble and insoluble fractions calculated from the concentrations in the expressed sap and press cakes represent soluble and insoluble amounts present in the living leaves with complete accuracy. It is necessary first to present the soluble and insoluble components found representing the phosphate and carbonate in the forms in which they could occur at the observed reactions of the expressed sap. The errors introduced during preparation of the material will then be considered in interpreting these results. Accordingly proportions of soluble monovalent and divalent phosphate are estimated from the observed pH values by use of the Brönstead-Hasselback equation employing the dissociation exponent $pK_2 = 7.20$ (**2**). This procedure is justified on the basis that the proportions of monovalent and divalent forms differ considerably in the range of reactions observed. In any case, it will be noted (tables IV and V) that the quantity of phosphate in the soluble phase was not of an order of magnitude which would influence the total soluble anion values significantly.

With respect to the carbonate system, the total carbon dioxide evolved from the expressed sap after acidification has been calculated as bicarbonate which is the dominant form in the observed pH range. In this case, too, it may be noted that the magnitude of these values is not great relative to other constituents and that in no case can the resultant totals be very significantly altered by this procedure for estimating bicarbonate. Ammonium ion concentrations were so low in the guayule leaves (**6**) that an account of them is unnecessary for the balance sheets.

Insoluble carbonate and phosphate are expressed as divalent ions. This seems justified by the low solubilities of the calcium salts of these acids as well as by the observation that aqueous suspensions of the press cakes had reactions of approximately pH 8.5 for all types of leaf samples. In cases where press cakes were farther extracted, suspensions of still more alkaline reaction were obtained. With respect to phosphorus these estimates are high to the extent of phosphorus compounds other than inorganic phosphate.

TABLE III

REACTION OF THE EXPRESSED SAP

TREATMENT	MATURE LEAVES		EXPANDING LEAVES	
	$(H^+) \times 10^7$	pH	$(H^+) \times 10^7$	pH
K_1	2.13	6.67	1.44	6.84
K_2	0.72	7.14	0.63	7.20
K_3	0.44	7.36	0.31	7.51
NaK_1	1.16	6.94	0.78	7.11
NaK_2	0.46	7.34	0.36	7.44
NaK_3	0.26	7.58	0.28	7.55
DRFS* 0.05	0.67	.....	0.50	.....
0.01	0.87	.....	0.70	.....

* Difference required for significance.

The reactions of the expressed sap are shown in table III. The observed pH values were converted to hydrogen ion concentrations of which the mean values are shown. The mean pH values reported were derived from these mean hydrogen ion concentrations. The pH values from low potassium leaves were consistently lower than those from other treatments in each series and in each age group of leaf samples. The high potassium leaves yielded average pH values consistently higher than those from other treatments, but these differences were not significant statistically. At each level of potassium supply, treatments of series 2 yielded higher pH values than did those of series 1 in each age group of leaves. The pH of sap expressed from expanding leaves was usually higher than that from mature leaves of the same treatment.

In tables IV and V individual and total inorganic cations and anions are partitioned into soluble and insoluble components of mature leaves and expanding leaves respectively. All constituents are expressed as milliequiva-

TABLE IV

INORGANIC CATION AND ANION COMPOSITION OF MATURE LEAVES

TREATMENT	MILLIEQUIVALENTS PER 100 GRAMS OF DRY TISSUE										MILLIEQUIVALENTS PER KILOGRAM OF FRESH TISSUE	
	K^+	Na^+	Ca^{++}	Mg^{++}	TOTAL CATIONS	$H_2PO_4^-$	$HPO_4^=$	$SO_4^=$	NO_3^-	TOTAL INORGANIC ANIONS	TOTAL CATIONS	TOTAL INORGANIC ANIONS
SAP SOLUBLE												
K_1	36	2	141	79	258	1.4	0.9	75	44	121	516	242
K_2	138	1	81	56	276	0.8	1.3	94	60	156	492	278
K_3	256	0	38	27	321	0.8	2.3	136	62	201	536	336
NaK_1	65	16	74	108	263	1.5	1.7	90	39	132	495	248
NaK_2	162	17	43	73	295	0.8	2.2	109	50	162	525	288
NaK_3	273	10	26	35	344	0.7	3.2	132	58	194	569	320
DRFS*												
0.05	6.6	3.8	12.1	6.8	18.1	0.35	0.55	11.5	10.7	21.2	34.1	18.1
0.01	9.4	6.3	17.2	9.6	25.7	0.50	0.78	16.3	15.2	30.2	48.4	25.8
INSOLUBLE												
K_1	.....	.....	218	17	235	.....	14	57	.....	71	471	142
K_2	.....	.....	208	17	225	.....	11	56	.....	67	401	119
K_3	.....	.....	124	15	139	.....	8	40	.....	48	232	80
NaK_1	.....	.....	190	31	221	.....	14	60	.....	74	416	139
NaK_2	.....	.....	170	26	196	.....	11	54	.....	65	349	116
NaK_3	.....	.....	90	19	109	.....	10	44	.....	54	180	89
DRFS*												
0.05	.....	.....	19.7	7.1	23.7	.....	1.4	8.1	.....	8.5	38.1	18.8
0.01	.....	.....	27.6	10.0	33.8	.....	2.0	11.5	.....	12.1	54.1	26.7
TOTAL												
K_1	36	2	359	96	493	1.4	15	132	44	192	987	384
K_2	138	1	289	73	501	0.8	12	150	60	223	893	397
K_3	256	0	162	42	460	0.8	10	176	62	249	768	416
NaK_1	65	16	264	139	484	1.5	15	150	39	206	911	387
NaK_2	162	17	213	99	491	0.8	13	163	50	227	874	404
NaK_3	273	10	116	54	453	0.7	13	176	58	248	749	410
DRFS*												
0.05	6.6	3.8	21.5	7.4	31.4	0.35	2.5	12.9	10.7	21.9	46.6	n.s.
0.01	9.4	6.3	30.6	10.5	44.7	0.50	3.6	18.4	15.2	31.1	66.3	.....

* DRFS = Difference required for significance.

TABLE V

INORGANIC CATION AND ANION COMPOSITION OF EXPANDING LEAVES

TREATMENT	MILLIEQUIVALENTS PER 100 GRAMS OF DRY TISSUE										MILLIEQUIVALENTS PER KILOGRAM OF FRESH TISSUE	
	K^+	Na^+	Ca^{++}	Mg^{++}	TOTAL CATIONS	$H_2PO_4^-$	$HPO_4^=$	$SO_4^=$	NO_3^-	TOTAL INORGANIC ANIONS	TOTAL CATIONS	TOTAL INORGANIC ANIONS
SAP SOLUBLE												
K_1	38	1	98	68	205	0.9	0.8	65	51	118	383	220
K_2	106	1	53	46	206	0.7	1.4	72	55	129	386	242
K_3	230	2	25	25	282	0.9	3.8	113	67	185	483	317
NaK_1	69	10	59	85	223	1.5	2.4	75	51	130	402	235
NaK_2	121	13	35	61	230	0.8	2.4	85	53	141	425	261
NaK_3	228	9	26	35	298	1.1	5.0	116	69	191	490	314
DRFS*												
0.05	14.8	1.8	10.5	11.4	25.9	0.37	0.91	12.8	8.5	17.8	27.7	24.0
0.01	21.1	2.9	15.0	16.1	36.8	0.52	1.30	18.2	12.0	25.6	39.3	34.1
INSOLUBLE												
K_1	.....	.....	191	16	207	.....	17	55	.....	72	386	134
K_2	.....	.....	177	23	200	.....	15	43	.....	58	375	109
K_3	.....	.....	97	19	116	.....	11	30	.....	41	199	70
NaK_1	.....	.....	147	28	175	.....	15	44	.....	59	316	107
NaK_2	.....	.....	138	23	161	.....	14	39	.....	53	298	98
NaK_3	.....	.....	74	16	90	.....	9	21	.....	30	148	49
DRFS*												
0.05	.....	.....	11.5	6.3	12.1	.....	3.3	9.9	.....	11.5	27.7	19.1
0.01	.....	.....	16.3	9.0	17.2	.....	4.7	14.0	.....	16.4	39.4	27.2
TOTAL												
K_1	38	1	289	84	412	0.9	18	120	51	190	769	354
K_2	106	1	230	69	406	0.7	16	115	55	187	761	351
K_3	230	2	122	44	398	0.9	15	143	67	226	682	387
NaK_1	69	10	206	113	398	1.5	17	119	51	189	718	342
NaK_2	121	13	173	84	391	0.8	16	124	53	194	723	359
NaK_3	228	9	100	51	388	1.1	14	137	69	221	638	363
DRFS*												
0.05	14.8	1.8	14.7	6.7	n.s.	0.37	n.s.	13.6	8.5	18.3	40	24.5
0.01	21.1	2.9	20.9	9.5	.....	0.52	.....	19.4	12.0	26.0	57	34.8

* DRFS = Difference required for significance.

lents per 100 grams of dry tissue. In the last two columns of each table totals for cations and anions are expressed as milliequivalents per kilogram of fresh material. With respect to soluble and insoluble fractions the outstanding differences between treatments are common to both bases of expression.

In the mature leaves total soluble cations increased progressively with increasing potassium in both series. On a dry weight basis differences were highly significant except in the comparison between treatments K_1 and K_2. Differences in individual cation contents were considerable. In each series deficiency of potassium was compensated for in part by increases in calcium and magnesium. Calcium predominated in the K_1 treatment; whereas, magnesium predominated in the NaK_1 treatment. In comparisons between intermediate and high potassium levels, high potassium contents were not nearly balanced by decreased soluble calcium and magnesium contents.

In the expanding leaves total soluble cations were essentially identical in K_1 and K_2 treatments and did not differ significantly between NaK_1 and

TABLE VI

CORRELATION COEFFICIENTS BETWEEN TOTAL INORGANIC CATIONS AND INORGANIC ANIONS IN SOLUBLE AND INSOLUBLE FRACTIONS*

	MATURE LEAVES	EXPANDING LEAVES
Soluble	+0.877	+0.947
Insoluble	+0.819	+0.885
Total	+0.845	+0.750

* Coefficients required for significance for 18 observations—0.05 = .468; 0.01 = .590.

NaK_2, i.e., in low potassium treatments decreases in potassium were compensated for by increases in calcium and magnesium in the expanding leaves.

Treatment variations in total soluble inorganic anions resulted largely from variations in soluble sulphate and nitrate in both mature and expanding leaves. Soluble sulphate increased in a rather regular fashion with increasing potassium concentrations particularly in the mature leaves. This trend was related in part to an increase in total sulphur content of the leaves and to a greater absorption of the sulphate ion by the whole plant at high potassium levels (**5**), but at the high potassium levels it was also associated with significant decreases in the insoluble sulphur content. With respect to variations in nitrate content differences in rates of nitrate reduction in the various treatments as well as differences in rates of absorption seem to be involved (**5**, **6**).

One feature shown by the data of tables IV and V is the general inverse relationship between soluble and insoluble components. Also evident is the close correlation between inorganic cations and inorganic anions in soluble and insoluble fractions in both mature and expanding leaves. Coefficients of these correlations are recorded in table VI.

* DRFS = Difference required for significance.

TABLE VII

EXCESS CATIONS, ORGANIC ACIDS AND CARBONATE COMPONENTS

TREATMENT	MILLIEQUIVALENTS PER 100 GRAMS OF DRY TISSUE									
	MATURE LEAVES					EXPANDING LEAVES				
	EXCESS CATIONS	ORGANIC ACIDS	HCO_3^-	$CO_3^=$	ORGANIC ACIDS + HCO_3^- + $CO_3^=$	EXCESS CATIONS	ORGANIC ACIDS	HCO_3^-	$CO_3^=$	ORGANIC ACIDS + HCO_3^- + $CO_3^=$
SAP SOLUBLE										
K_1	137	99	6	.....	105	87	64	13	.....	77
K_2	120	95	12	.....	107	77	63	21	.....	84
K_3	120	97	17	.....	114	97	76	26	.....	102
NaK_1	131	93	7	.....	100	93	67	14	.....	81
NaK_2	133	83	16	.....	99	89	59	24	.....	83
NaK_3	150	94	14	.....	108	107	85	25	.....	110
DRFS*										
0.05	19.5	13.7	4.4	.....	n.s.	14.6	8.8	4.3	.....	10.2
0.01	27.7	19.4	6.3	.....	.....	20.8	12.6	6.2	.....	14.6
INSOLUBLE										
K_1	164	33	.....	125	158	135	16	.....	94	110
K_2	158	32	.....	127	159	142	16	.....	100	116
K_3	91	26	.....	63	89	75	16	.....	24	40
NaK_1	147	33	.....	106	139	116	18	.....	75	93
NaK_2	131	30	.....	98	128	108	16	.....	68	84
NaK_3	55	24	.....	42	66	60	17	.....	7	24
DRFS*										
0.05	22.9	3.5	.....	13.0	22.3	16.2	n.s.	.....	11.7	13.0
0.01	32.5	5.0	.....	18.5	31.7	23.1	.....	.....	16.6	18.5
TOTAL										
K_1	301	132	6	125	263	222	80	13	94	187
K_2	278	127	12	127	266	219	79	21	100	200
K_3	211	123	17	63	203	172	92	26	24	142
NaK_1	278	126	7	106	239	209	85	14	75	174
NaK_2	264	113	16	98	227	197	75	24	68	167
NaK_3	205	118	14	42	174	167	102	25	7	134
DRFS*										
0.05	22.7	n.s.	4.3	13.0	19.8	18.2	8.8	4.3	11.7	15.2
0.01	32.3	.....	6.3	18.5	28.1	25.8	12.6	6.2	16.6	21.6

* Difference required for significance.

In table VII are shown values for the excess of cations over inorganic anions, total non-volatile organic acids and carbonate in mature and expanding leaves. In each case the total excess of cations was lower in high potassium treatments (K_3 and NaK_3) than in other treatments. It may be seen that these variations were brought about entirely by corresponding variations in the insoluble fraction, which consisted largely of calcium and carbonate. In the soluble fraction from the mature leaves no consistent variations in excess cations were apparent. In NaK_3, the excess of cations was significantly higher than in K_2 or K_3, but these differences were not associated with any corresponding differences in organic acids. The soluble fraction from the expanding leaves showed high values for excess cations in the high potassium treatments. These high values were associated with high organic acid contents. Correlation coefficients for the relationship between excess cations and several anion fractions are shown in table VIII. The outstanding relationship was that between carbonate

TABLE VIII

CORRELATION COEFFICIENTS OF RELATIONSHIPS BETWEEN EXCESS CATIONS AND ORGANIC ANION FRACTIONS*

	MATURE LEAVES	EXPANDING LEAVES
Soluble cation excess and soluble organic acids	−0.116	+0.769
Insoluble cation excess and carbonate plus insoluble organic acids	+0.955	+0.946
Total cation excess and organic acids plus carbonate	+0.846	+0.750
Insoluble calcium and carbonate	+0.984	+0.979

* Coefficients required for significance for eighteen observations: 0.05 = 0.468; 0.01 = 0.590.

and the excess cations of the insoluble fractions (largely calcium). In the expanding leaves a lower, but highly significant correlation was shown between soluble organic acids and soluble excess cations. In the mature leaves there was no such correlation.

INDIVIDUAL ORGANIC ACIDS

In table IX individual and total sap soluble organic acids are reported as milliequivalents per 100 grams of dry leaf tissue and as milliequivalents per liter of sap. In the mature leaves most of the treatment variations in the sap concentration of total organic acids were associated with variations in total dry weight of the leaves. The result is, higher sap concentrations in low potassium leaves were not significant on the dry weight basis. However, some of the variations in malic and citric acids were considerable, and most of these variations are evident from either basis of expression.

MALIC ACID.—In the mature leaves the principal variations in sap concentration of total organic acids were associated with variations in malic

* Difference required for significance.

acid. At low and intermediate potassium levels the concentration of malic acid was lower in series 2 than in series 1. The concentration of this acid was closely related to the concentration of soluble calcium as shown in figure 1. The correlation coefficient is +0.877. The treatments may be divided rather sharply into two groups according to malic acid contents; i.e., treatments K_1, K_2, and NaK_1, had relatively high concentrations, whereas K_3, NaK_2, and NaK_3 were relatively low in this acid. These two groups of treatments had relatively high and low calcium contents respectively. For any given treatment, malic acid was much lower in expanding leaves than

TABLE IX

SAP SOLUBLE ORGANIC ACIDS

TREATMENT	MILLIEQUIVALENTS PER 100 GRAMS DRY TISSUE				MILLIEQUIVALENTS PER LITER OF SAP				UNKNOWN ACIDS AS % OF TOTAL
	MALIC	CITRIC	UNKNOWN ACIDS	TOTAL	MALIC	CITRIC	UNKNOWN ACIDS	TOTAL	
MATURE LEAVES									
K_1	55	25	18	99	132	59	47	238	19
K_2	50	20	25	95	104	42	51	197	26
K_3	29	30	36	97	56	56	73	185	37
NaK_1	42	21	30	93	94	44	68	206	32
NaK_2	29	21	33	83	61	42	70	173	40
NaK_3	27	28	37	92	51	57	71	179	40
DRFS*									
0.05	7.1	5.9	9.8	13.7	14.3	12.5	17.6	23.4	.....
0.01	10.1	8.4	13.9	19.4	20.4	17.8	25.0	33.3	.....
EXPANDING LEAVES									
K_1	27	15	22	64	59	33	43	135	34
K_2	18	11	34	63	40	25	69	134	54
K_3	18	25	33	76	36	50	58	144	43
NaK_1	18	11	38	67	38	23	75	136	57
NaK_2	13	13	33	59	29	28	66	123	56
NaK_3	17	24	44	85	32	52	69	153	52
DRFS*									
0.05	7.5	3.3	10.7	8.8	14.8	6.3	24.1	14.1	.....
0.01	10.7	4.7	15.2	12.6	21.0	9.0	34.3	20.1	.....

* Difference required for significance.

in mature leaves. In the expanding leaves, malic acid was increased only by the K_1 treatment. Among the expanding leaf samples this treatment also had the highest calcium content.

CITRIC ACID.—No such simple relationship can be shown between any single cation and the concentration of citric acid. It is noteworthy, however, that in both mature and expanding leaves, and in each series, this acid was significantly higher in the high potassium treatment than at the intermediate potassium level. These differences were all statistically significant, but were particularly outstanding in the expanding leaves. However, citric acid also increased in the leaves of potassium deficient plants in series 1 (K_1).

UNKNOWN ACIDS.—In the mature leaves these acids were lower in treatments K_1 and K_2 than in either of the high potassium treatments. In K_1, the concentration of this group of acids was lower than in any of the treatments of series 2. In the expanding leaves, K_1 had lower concentrations than any other treatment, whereas NaK_3 had a higher content than treatments other than K_2 and NaK_1. Attempts to detect oxalic acid in guayule leaves were without success.

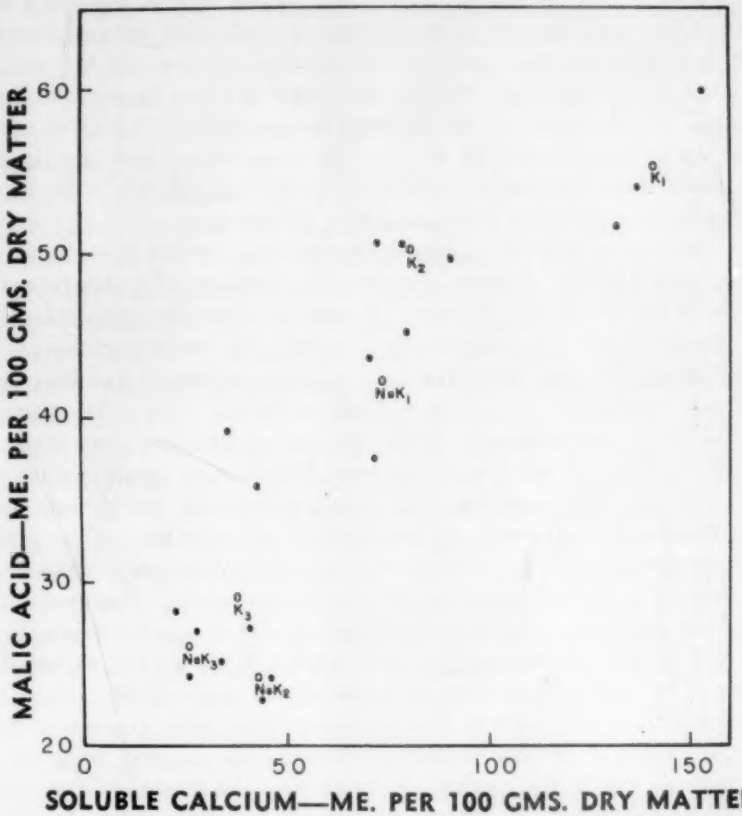

FIG. 1. Relationship between sap soluble calcium and malic acid in mature leaf samples. Small dots represent samples from individual plots. Circles denote treatment means. Coefficient of correlation = + 0.877. Required for significance, 0.590 ($n = 18$, $p = 0.01$).

Discussion

In the leaves of guayule the excess of cations over inorganic anions is not nearly balanced by the ether soluble organic acids. In this respect guayule differs from the tobacco plant (**22**) and from eleven of the twelve species studied by PIERCE and APPLEMAN (**21**). However, the results for guayule are in agreement with those of the latter workers for cantaloupe. Guayule and cantaloupe differ from the other species studied by Pucher

et al., and Pierce and Appleman in that the leaves contain no oxalate. In guayule, the discrepancy between excess cations and organic acids is accounted for by carbonate. The sum of the total organic acids and carbonate is highly correlated with the excess of cations over inorganic anions, throughout the range of nutrient treatment employed.

The dominant position of insoluble carbonate in the cation-anion balance of guayule leaves is of special interest. There can be little doubt that the carbonate is very largely, if not entirely, in the form of calcium carbonate. In addition to the very high correlation coefficients between insoluble calcium and insoluble carbonate is the fact that calcium was the only cation present in sufficient quantity in the insoluble phase to account for the carbonate. Furthermore, in the mature leaves (table VII) after correcting for the smaller quantities of insoluble magnesium and the insoluble anions other than carbonate, quantities of residual calcium were in very close numerical agreement with quantities of carbonate.

The presence of calcium carbonate deposits in the cells of certain plants has long been known. Meyen, according to DeBary (**7**), discovered the cystoliths of the Urticaceae in 1839. A number of species from other families of plants contain less conspicuous deposits. In the Boraginaceae, Cruciferae, Compositae and other families, calcium carbonate has been found in or closely associated with the epidermal hair cells. Yet, in the numerous studies of tissue reactions and buffer systems which have been made, the carbonate system has not frequently been found to be an important component. Small (**29**) emphasized the errors which may result from loss of carbon dioxide from the tissues, particularly when extracted or pressed. He also mentioned calcium carbonate as one of the insoluble calcium salts which may serve a buffering function by precipitation. However, in the tissues from numerous species investigated by Small and his students, pH values of 7 or above were rarely encountered. Martin (**17**) reported the presence of a carbonate system in stems of the bean plant. Haas (**11**) obtained alkaline pH values in sap expressed from sweet clover and determined the volume of carbon dioxide which could be liberated from the sap.

In guayule leaves, histological study showed the presence of carbonate in the epidermal trichomes, but no evidence of its presence in other tissues could be found. This conforms to the observations reviewed by DeBary in that calcium carbonate was frequently found in the epidermal hairs or otherwise closely associated with the epidermis. The results of Thoday and Evans (**31**) are of interest in this connection. Various solutes were found to be rather highly localized in different tissues or cells. The most striking example was in Mesembryanthimum where some cells of the water tissue contained abundant calcium in solution whereas other cells contained soluble oxalate.

In the experiment concerned with the recovery of carbonate from guayule leaf tissues, it was found that rather large variations in pH of the sap expressed from a given leaf sample resulted from variations in the time

allowed for thawing of the frozen material. The pH values increased as the time of thawing was prolonged (table II). Parallel with increasing pH was an increase in carbonate constituents of the sap (largely bicarbonate in this range of reaction). Another result of prolonged standing of the thawed tissues was a decrease in the phosphorus content of the sap. This decrease was accounted for by increased insoluble phosphorus. From these observations it is suggested that the observed bicarbonate and pH values of expressed sap result from a reaction of calcium carbonate with uncombined acids. These reactions take place after a composite mixture of the tissue fluids has resulted from disorganization of the protoplasmic structure by freezing and thawing. It must be considered that in the living state the carbonate is largely localized in the epidermal trichomes and that the reactions of the other cells of the leaf may be considerably more acid than is the reaction of the expressed sap.

It is of interest to apply this interpretation to the analytical results reported in tables IV, V, and VII in an attempt to reconstruct the distribution prevailing in the living leaves. In accordance with this interpretation, the total phosphorus found in the leaves is expressed as $H_2PO_4^-$. The "bicarbonate" reported in table VII is considered to have arisen from insoluble carbonate. Approximations relative to the forms of other ions are the same as previously. The results of these calculations are shown in table X. It is evident that the relationships shown in table VII are not qualitatively altered and are as significant when expressed in this manner. It may be noted that the numerical agreement between excess cations and organic acids plus carbonate is improved by this mode of expression.

In table X it is noted that the ratio: excess cations/organic acids plus carbonate exceeded unity in all cases except one, indicating a general excess of cations over all anions accounted for. Variations in this ratio indicate this excess was greater in the low potassium samples. However, the pH values were less than 7 for sap expressed from mature and expanding leaves of K_1 and from mature leaves of NaK_1 (table III). Furthermore, the evidence as noted above indicates that these values were somewhat higher than those of the living cells of the mesophyll. At this point it is necessary to recall that no account has been taken of the calcium, magnesium, sulphur, and phosphorus combined in forms other than salts of inorganic acids, non-volatile organic acids and carbonate. It appears that in the low potassium treatments the proportion of cations and anions combined in forms other than those considered was altered. The altered ratio in the low potassium samples could be accounted for by greater amounts of calcium or magnesium, or smaller amounts of sulphur or phosphorus in organic combination. In tobacco leaves (**22**) it was similarly found that the ratio: excess cations/organic acids exceeded unity, yet the reaction of these tissues indicated the organic acids are not entirely combined. Although there was correlation between excess cations and organic acids in tobacco leaves, the ratio as in guayule leaves was altered with changing mineral contents of the samples.

The proportions of individual acids present in potassium deficient leaves of series 1 were different from those in leaves containing adequate potassium. The expanding leaves of K_1 contained higher concentrations of malic and citric acids and lower concentrations of unknown acids in comparison with expanding leaves of K_2. Similar variations were indicated in the mature leaves of these plants although these were statistically significant only when expressed in terms of sap concentration. Such variations were not apparent in series 2. These results may suggest that the accu-

TABLE X

CATION-ANION BALANCE OF MATURE AND EXPANDING LEAVES

TREATMENT	TOTAL CATIONS	TOTAL INORGANIC ANIONS	EXCESS CATIONS	ORGANIC ACIDS	CARBONATE	ORGANIC ACIDS + CARBONATE	RATIO: EXCESS CATIONS / ORGANIC ACIDS + CARBONATE*
	MILLIEQUIVALENTS PER 100 GRAMS DRY MATTER						
MATURE LEAVES							
K_1	493	185	308	132	137	269	1.15
K_2	501	217	284	127	151	278	1.02
K_3	460	244	216	123	97	220	.98
NaK_1	484	198	286	126	120	246	1.16
NaK_2	491	220	271	113	130	243	1.12
NaK_3	456	241	215	118	70	188	1.14
DRFS†							
0.05	31.4	22.0	24.0	n. s.	10.4	17.0	.....
0.01	44.7	31.3	34.1	.....	14.8	24.1	.....
EXPANDING LEAVES							
K_1	413	181	231	80	120	200	1.16
K_2	406	179	227	79	142	221	1.03
K_3	398	218	180	92	76	168	1.07
NaK_1	398	180	218	85	103	188	1.16
NaK_2	391	186	205	75	116	191	1.07
NaK_3	388	214	174	102	57	159	1.09
DRFS†							
0.05	n. s.	16.8	23.3	8.8	13.6	19.2	.....
0.01	.....	23.9	33.2	12.6	19.3	27.4	.....

* Correlation coefficients: Excess cations and carbonate plus organic acids. Mature leaves + 0.839; Expanding leaves + 0.784.

† Difference required for significance.

mulation of malic and citric acids in leaves of K_1 is in some way related to the high soluble calcium contents of these leaves. ILJIN (**14**), according to PIERCE and APPLEMAN (**21**), found in general that soluble calcium in plants was highly correlated with malic and citric acids. Pierce and Appleman in comparing different species of plants grown in the same nutrient solution found malic and citric acids to be somewhat correlated with soluble calcium, particularly in the leaves. VICKERY *et al.*, (**34**) grew tobacco plants in solutions containing various proportions of nitrate and ammonium nitrogen. Leaves of plants supplied with high percentage of nitrate contained high proportions of malic and citric acids, whereas those

of plants supplied with solutions containing mostly ammonium nitrogen had higher proportions of acids of the unknown group. Similar results have been obtained by WADLEIGH and SHIVE (**35**) for corn and by CLARK (**4**) for tomato plants. In ammonium culture, the content of total mineral cations including calcium is relatively low. In the present experiment, malic acid of the mature leaves seemed to be related to soluble calcium content throughout the range of treatments. Citric acid in some cases increased in high calcium leaves, but greater and more consistent increases were found in leaves very low in calcium. It should be noted, however, that the plants used in this experiment were relatively young. It is possible that the proportions of individual acids would be altered with age, or even by diurnal variations.

It was not possible to determine whether the hydrogen ion concentration of the leaf cells was altered by potassium deficiency or what effect this treatment had on the proportions of free and combined acids in these cells. However, any alterations in tissue reactions which may have occurred in the potassium deficient leaves could not have resulted from decrease in the excess of total soluble cations, since there was no decrease in these values, nor in the total soluble organic acids. These results differ from some of the results reported by DUNNE (**8**) for other plants. Plants which do not normally contain large amounts of soluble calcium such as wheat and buckwheat may differ considerably in their response to potassium deficiency, in that sufficient soluble calcium may not be present to maintain sufficient excess of soluble cations. In such cases, a reduction in concentrations of components necessary for the buffer system may be one of the early results of potassium deficiency.

Leaves of high potassium plants invariably had low values for the excess of total cations over inorganic anions. These low values were correlated with low contents of insoluble carbonate, which in turn were directly related to the low calcium contents of these tissues; nevertheless, the total soluble cation and inorganic anion contents of these tissues were invariably higher. In the mature leaves consistent variations in the excess of soluble cations were not found. In the expanding leaves of high potassium plants there were increases in the excess of soluble cations over soluble inorganic anions. These increases were correlated with increases in soluble organic acids. This result is analogous to results obtained by ULRICH (**33**) in that an increase in soluble cations in excess of soluble inorganic anions was associated with an increase in organic acids. DUNNE (**8**) found that wheat and buckwheat plants supplied with low calcium solutions had higher values for the excess of soluble cations. The sap from these plants showed increased buffering in the acid range, indicating higher organic acid content. The increase in organic acids was apparently great enough to compensate for the higher cation contents in the sap, since the pH of the sap was the same for plants of high and low calcium contents. This is of interest in connection with the results from guayule, since in guayule leaves it was

not possible to determine whether the reactions of the fluids within the tissue were actually altered.

Summary

1. Cation-anion relationships were studied in expanding and mature leaves of guayule plants which had been supplied with low, intermediate, and high levels of potassium. It was found that the excess of cations over inorganic anions in these leaves was greatly in excess of the non-volatile organic acids present.

2. Highly significant correlations were found for a relationship between the non-volatile organic acids plus carbonate and the excess of cations over inorganic anions. However, this relationship was dependent largely upon variations in insoluble calcium and carbonate.

3. A large proportion of the calcium present in the leaves was insoluble. The insoluble calcium content was closely related to the insoluble carbonate content. The carbonate was highly localized in the epidermal hairs. No oxalate could be found in the leaves.

4. The recovery of carbonate and the distribution of components of the carbonate system between soluble and insoluble phases were compared after subjecting samples to several treatments.

5. Total organic acids and values for excess cations in the leaves were at least as high in low potassium as in intermediate potassium treatments of the same series. There was a greater excess of cations over all anions accounted for in low potassium than in other treatments.

6. In the expanding leaves there was a highly significant correlation between sap soluble organic acids and the excess of sap soluble cations throughout the range of treatments. The principal treatment variations were higher contents of organic acids and excess cations in leaves from high potassium treatments. The higher total organic acid contents of high potassium leaves were largely accounted for by higher contents of citric acid, although in one case the acids of the unknown group also appeared to be involved. In the mature leaves consistent variations in sap soluble organic acids or in excess of soluble cations were not found.

7. In the mature leaves, sap soluble calcium was highly correlated with sap soluble malic acid. Malic acid was generally much lower in expanding leaves than in mature leaves of the same plants, but in expanding leaves too, the highest malic acid content was found in leaves containing the most calcium.

Hawaii Agricultural Experiment Station
Honolulu, Hawaii

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EFFECTS OF POTASSIUM DEFICIENCY AND EXCESS UPON CERTAIN CARBOHYDRATE AND NITROGENOUS CONSTITUENTS IN GUAYULE[1]

BRUCE J. COOIL[2] AND M. C. SLATTERY[2]

(WITH TWO FIGURES)

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The nature of the carbohydrates of translocation and the reserve carbohydrates in guayule has only recently been elucidated. McRARY and TRAUB (**28**) isolated a fructosan, tentatively identified as inulin, and HASSID *et al.* (**19**) definitely characterized this substance as inulin. Later, TRAUB and SLATTERY (**41**) showed that in addition to inulin, levulins are also present in guayule tissue, and in relatively greater amounts. TRAUB and SLATTERY (**41**) also indicated the importance of relating the 89 per cent. ethanol soluble and insoluble levulins to plant functioning. TRAUB, SLATTERY and WALTER (**42**) have shown that the carbohydrates of translocation in guayule are apparently the free reducing sugars since they are always present in relatively small amounts in the roots, stems and branches but predominate in the leaves. These workers have also shown that of the total free reducing sugars, the amounts of free non-fructose sugars are always greater than the amounts of free fructose in spite of the fact that the chief reserve carbohydrates in guayule are polymers of fructose, levulins and inulin. The conditions of the present experiment were not conducive to large accumulations of inulin, or higher polymer levulins, insofar as the experiment consisted of a phase of rapid growth.

So far as the writers are aware, no detailed study has been made of the nitrogenous fractions in guayule.

Effects of potassium supply upon growth responses and mineral contents of guayule plants and upon cation and inorganic and organic anion contents of the leaves have been reported (**8, 9**). In this paper are considered carbohydrate and nitrogenous fractions in tissues of the same plants. Carbohydrate fractions in plants grown with and without potassium are also reported.

Methods

The methods employed in growing the plants have been described (**8**). Treatments designated K_1, K_2 and K_3 comprised series 1. The nutrient

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[2] Formerly Assistant Physiologists, Special Guayule Research Project.

solutions for these treatments contained 0.5, 3 and 12 milli-equivalents K per liter, respectively. These solutions each contained 10 m.e. Ca per liter and no Na except as impurities. Treatments designated NaK_1, NaK_2 and NaK_3 comprised series 2. The solutions supplied to these plants contained the same concentrations of K as the corresponding solutions of series 1, but each solution contained 4 m.e. Ca and 6 m.e. Na per liter. In a concurrent experiment, nursery-grown plants from the same lot received solutions with and without potassium. The solutions employed were the complete solution containing nitrate as the only source of nitrogen and the minus potassium solution suggested by HOAGLAND and ARNON (**21**). The treatments are correspondingly designated + K and − K. Plants of the latter experiment received nutrient treatments from December 23, 1944, until April 24, 1945. The methods of growing and harvesting the plants were otherwise the same as in the main experiment.

Reducing sugars, levulins and inulin were determined as described by TRAUB and SLATTERY (**40**). Total nitrogen was determined in dried samples by a modification of the procedure of PEPKOWITZ and SHIVE (**32**) described by KELLEY *et al.* (**23**). Ammonia, α-amino and total amide nitrogen were estimated by the procedures of SCHLENKER (**37**). For total soluble nitrogen, a 5-ml. aliquot of sap was heated to 70° C., cooled, centrifuged and the precipitate washed twice with 5 ml. portions of water, centrifuging after each washing. The supernatant liquid and washings were made to a volume of 50 ml. A 10-ml. aliquot of the dilute sap was transferred to a 100-ml. volumetric flask. The nitrate was carefully reduced with reduced iron powder essentially as described by PUCHER, LEAVENWORTH and VICKERY (**33**). Three ml. of sulphuric acid (1 plus 1) were added. After mixing, 1 gram of reduced iron powder was introduced. The flask was shaken for ten minutes, then gently heated to boiling on a gas hot plate, boiled five minutes and cooled. Subsequent digestion was accomplished by a modification of the procedure of PEPKOWITZ and SHIVE (**32**) as adapted to analysis of guayule tissue by KELLEY *et al.* (**23**). Five ml. of concentrated sulphuric acid containing 2.4 gm. selenium oxichloride per liter were introduced. The flask was heated gently until the water was expelled and finally digested until clear. The solution was then cooled and made to volume with distilled water. Aliquots of the digested solution were made alkaline with sodium hydroxide, aspirated into dilute sulphuric acid and nesslerized. The Nessler's reagent was prepared by the procedure of FRAPS and STERGES (**11**). The residue from the sap which had been heated, cooled and centrifuged, was dissolved in concentrated sulphuric acid and digested until clear. The digest was cooled, made to volume, and an aliquot nesslerized. In all samples this fraction was very low and no significant variations resulted from nutrient treatments. Therefore, in presenting the results, the sap soluble coagulable nitrogen has been added to the insoluble nitrogen fraction. Soluble organic nitrogen was calculated from the total soluble nitrogen by correcting for nitrate and ammonium nitrogen.

Results

Carbohydrate fractions

Total carbohydrate contents of the plants are shown in table I. When expressed in terms of grams per plant, no significant variation in total carbohydrates resulted from treatment variations within a series. The contents in series 1, however, were consistently higher than those in series 2. In part, these differences resulted from the smaller plants in any given potassium treatment of series 2. In terms of milligrams per gram of dry weight, the K_1 treatment had a significantly higher concentration of total carbohydrates than did any of the other treatments. Other differences between individual treatments were not statistically significant, although these values were generally lower in series 2 than in series 1.

TABLE I

Total carbohydrate contents* of plants receiving various nutrient treatments

Treatment	Grams per plant	Mg. per gram of dry weight
K_1	0.262	19.0
K_2	0.275	16.1
K_3	0.266	15.9
NaK_1	0.182	13.6
NaK_2	0.207	14.1
NaK_3	0.180	13.8
DRFS† 0.05	0.043	2.6
0.01	0.062	3.7

* Carbohydrates of leaves, stems and roots.
† Difference required for significance.

The carbohydrate compositions of individual tissues are shown in table II. In both expanding and mature leaves, total carbohydrates were highest in the low potassium treatments (K_1 and NaK_1). In the expanding leaves these accumulations are accounted for entirely in terms of reducing sugars. In the mature leaves of the K_2 plants inulin was higher than in the mature leaves of plants of other treatments. In the young stems total carbohydrates and reducing sugars were significantly higher in K_1 than in other treatments. In these tissues the relatively high variability in levulins does not permit close comparison.

In the old stems, total carbohydrates were evidently decreased by either low or high potassium treatments in series 1 (i.e., lower in K_1 or K_3 than in K_2). Analogous differences in series 2 were not statistically significant. In the roots, total carbohydrates were lower in K_1 than in K_2. In the old stems and roots, total carbohydrates were generally lower in series 2 than in series 1 at any given level of potassium. In these tissues, in contrast to the leaf and young stem tissues, variations in total carbohydrates resulted almost wholly from variations in levulins.

In column 5 of table II, reducing sugars are expressed as percentage of

TABLE II

CARBOHYDRATE FRACTIONS*

TREATMENT	TOTAL CARBOHYDRATES	INULIN	LEVULIN	REDUCING SUGARS	REDUCING SUGARS
	PERCENTAGE OF DRY WEIGHT				PERCENTAGE OF TOTAL CARBOHYDRATES*
EXPANDING LEAVES					
K_1	1.38	0.18	0.36	0.83	60.1
K_2	0.82	0.16	0.28	0.38	46.3
K_3	0.97	0.11	0.48	0.37	38.1
NaK_1	1.13	0.20	0.30	0.64	56.6
NaK_2	0.90	0.19	0.34	0.36	40.0
NaK_3	0.79	0.11	0.38	0.30	38.0
DRFS† 0.05	0.22	n.s.	n.s.‡	0.19	.....
0.01	0.31	.....	.....	0.26	.....
MATURE LEAVES					
K_1	1.97	0.19	0.44	1.35	57.9
K_2	1.25	0.34	0.35	0.57	45.6
K_3	1.22	0.21	0.59	0.42	34.4
NaK_1	1.53	0.21	0.46	0.86	56.2
NaK_2	1.25	0.22	0.51	0.51	40.8
NaK_3	1.12	0.14	0.55	0.43	38.4
DRFS† 0.05	0.27	0.11	0.21	0.34	.....
0.01	0.39	0.15	0.30	0.49	.....
YOUNG STEMS					
K_1	2.41	0.13	1.32	0.96	39.8
K_2	1.66	0.16	0.98	0.53	31.9
K_3	1.96	0.10	1.60	0.26	13.3
NaK_1	1.38	0.07	0.90	0.41	29.7
NaK_2	1.67	0.12	1.24	0.31	18.6
NaK_3	1.49	0.13	1.05	0.31	20.8
DRFS† 0.05	0.68	n.s.	n.s.	0.29	.....
0.01	0.96	.....	.....	0.40	.....
OLD STEMS					
K_1	1.95	0.19	1.32	0.43	22.1
K_2	2.40	0.25	1.71	0.44	18.3
K_3	1.87	0.15	1.29	0.43	23.0
NaK_1	1.27	0.15	0.77	0.35	27.6
NaK_2	1.67	0.14	1.17	0.36	21.6
NaK_3	1.51	0.12	1.05	0.35	23.2
DRFS† 0.05	0.42	n.s.	0.31	.....	.....
0.01	0.60	.....	0.45	.....	.....
ROOTS					
K_1	1.89	0.03	1.22	0.63	33.3
K_2	2.62	0.09	1.96	0.57	21.8
K_3	2.26	0.01	1.66	0.59	26.1
NaK_1	1.44	0.03	0.94	0.47	32.0
NaK_2	1.68	0.08	1.14	0.46	27.4
NaK_3	1.76	0.16	1.10	0.49	27.8
DRFS† 0.05	0.60	n.s.	0.48	0.12	.....
0.01	0.85	.....	0.68	0.17	.....

* Inulin, levulins and reducing sugars.
† Difference required for significance.
‡ No significant difference.

total carbohydrates. The interesting aspect of these data is the fact that in each plant part of the reducing sugars represent a higher percentage of the total carbohydrates in low potassium treatments than in intermediate potassium treatments in each series. The high proportion of reducing sugars found in the leaves of low potassium plants was due to increased concentrations of these sugars, rather than to variations of other fractions. In the old stems and roots of these plants, the high proportion of reducing sugars resulted from decreased concentrations of levulins.

TABLE III

CARBOHYDRATE CONTENT OF PLANTS GROWN WITH AND WITHOUT POTASSIUM*

TREATMENT	TOTAL CARBOHYDRATES†	INULIN	LEVULIN	REDUCING SUGARS	REDUCING SUGARS AS PER CENT OF TOTAL CARBOHYDRATES
		PERCENTAGE OF THE DRY WEIGHT			
		EXPANDING LEAVES			
−K	1.79	0.10	0.40	1.29	72.1
+K	1.22	0.26	0.42	0.54	44.3
		MATURE LEAVES			
−K	3.50	0.08	0.74	2.69	76.9
+K	1.42	0.09	0.55	0.79	55.6
		YOUNG STEMS			
−K	3.39	0.02	1.79	1.59	46.9
+K	3.11	0.11	2.54	0.46	14.8
		OLD STEMS			
−K	1.59	0.25	0.89	0.44	27.7
+K	2.53	0.31	1.76	0.46	18.2
		ROOTS			
−K	1.88	0.32	1.01	0.55	29.3
+K	2.77	0.08	2.20	0.49	17.7
		FLOWERS AND FRUITS			
−K	4.57	0.03	0.93	3.62	79.2
+K	5.11	0.08	1.04	4.00	78.3

* 17-month nursery plants received nutrient treatments from December 23, 1944, to April 24, 1945.

† Inulin plus levulins plus reducing sugars.

A concurrent experiment consisted of treatments where potassium was supplied or withheld (cf. methods). Carbohydrate contents of these plants are shown in table III. As in the previous experiment, the results reported are mean values from three replications.

The main effects of the low potassium treatments noted above were accentuated by withholding potassium. In the leaves greater total carbohydrate concentrations were found in the −K treatment than in the +K treatment. These differences may be largely attributed to higher reducing sugar concentrations in the leaves of −K plants. In the young stems of

TABLE IV

NITROGENOUS FRACTIONS

TREATMENT	TOTAL N	SAP INSOLUBLE N	SAP SOLUBLE ORGANIC N	TOTAL ORGANIC N	AMIDE N	AMINO N	AMMONIUM N	NITRATE N
	PERCENTAGE OF DRY WEIGHT							
	MATURE LEAVES							
K_1	4.12	3.03	0.47	3.50	0.037	0.082	0.009	0.612
K_2	4.05	2.73	0.47	3.20	0.014	0.048	0.015	0.837
K_3	3.68	2.34	0.45	2.79	0.007	0.033	0.015	0.877
NaK_1	4.06	3.04	0 46	3.50	0.020	0.059	0.009	0.551
NaK_2	3.96	2.76	0.48	3.24	0.024	0.075	0.008	0.708
NaK_3	3.78	2.64	0.31	2.95	0.018	0.047	0.012	0.821
DRFS*								
0.05	0.24	0.29	0.10	0.28	0.009	0.021	0.004	0.150
0.01	0.35	0.41	0.14	0.40	0.013	0.029	0.005	0.213
	EXPANDING LEAVES							
K_1	5.15	3.82	0.61	4.43	0.035	0.119	0.009	0.713
K_2	5.04	3.74	0.53	4.27	0.017	0.056	0.008	0.766
K_3	4.45	3.10	0.40	3.50	0.013	0.047	0.009	0.944
NaK_1	5.14	3.87	0.55	4.42	0.027	0.068	0.008	0.714
NaK_2	4.86	3.63	0.48	4.11	0.019	0.054	0.009	0.739
NaK_3	4.27	3.01	0.29	3.30	0.012	0.061	0.008	0.966
DRFS*								
0.05	0.21	0.41	0.15	0.39	0.013	0.025	n.s.†	0.119
0.01	0.29	0.59	0.21	0.55	0.018	0.036	.....	0.168

* Difference required for significance.
† No significant difference.

– K plants reducing sugars were higher and levulins lower than in the + K plants. In the old stems and roots – K plants had lower concentrations of total carbohydrates resulting almost entirely from lower levulin contents. In each plant part the reducing sugars when expressed as percentage of total carbohydrates were higher in the – K than in the + K plants. Except in the flower samples these differences were relatively great. In the latter samples reducing sugars were very high in both treatments.

Nitrogenous fractions of the leaves

In table IV are shown the total nitrogen contents of the leaves, together with several nitrogen fractions expressed as percentage of the dry weight.

TABLE V

Nitrogenous fractions given as percentages of total nitrogen

Treatment	Sap insoluble N	Sap soluble organic N	Total organic N	Amide N	Amino N	Ammonium N	Nitrate N
			Mature leaves				
K_1	73.5	11.4	84.9	0.90	2.00	0.22	14.8
K_2	67.4	11.6	79.0	0.35	1.19	0.37	20.7
K_3	63.6	12.2	75.8	0.19	0.90	0.41	23.8
NaK_1	74.9	11.3	86.2	0.49	1.45	0.22	13.6
NaK_2	69.7	12.1	81.8	0.61	1.89	0.20	17.9
NaK_3	69.8	8.2	78.0	0.48	1.24	0.32	21.7
DRFS*							
0.05	4.4	3.41	4.13	0.23	0.56	0.10	3.6
0.01	6.2	4.84	5.87	0.32	0.80	0.15	5.2
			Expanding leaves				
K_1	74.2	11.8	86.0	0.68	2.31	0.17	13.8
K_2	74.2	10.5	84.7	0.34	1.11	0.16	15.2
K_3	69.7	8.9	78.6	0.29	1.06	0.20	21.2
NaK_1	75.3	10.7	86.0	0.53	1.32	0.16	13.9
NaK_2	74.7	9.9	84.6	0.39	1.11	0.19	15.2
NaK_3	70.5	6.8	77.3	0.28	1.43	0.19	22.6
DRFS*							
0.05	3.5	3.31	2.64	0.27	0.30	n.s.†	2.5
0.01	5.0	4.71	3.76	0.39	0.42	.....	3.6

* Differences required for significance.
† No significant difference.

These fractions are also presented in table V, where they are expressed as percentage of the total nitrogen content. The total nitrogen content of mature leaves was significantly lower in K_3 than in other treatments except NaK_3. The latter had significantly less total nitrogen than K_1, K_2 or NaK_1. In the expanding leaves lower total nitrogen contents were found in the two high potassium treatments than in others. In addition, NaK_2 was significantly lower in nitrogen than NaK_1.

Variations in sap insoluble nitrogen followed a trend similar to that of the total nitrogen content. In the mature leaves this fraction was signifi-

cantly lower in K_3 than in other treatments. In addition, NaK_3 was lower than K_1 or NaK_1. In the expanding leaves the sap insoluble nitrogen contents of K_3 and NaK_3 were lower than those of other treatments. When expressed in terms of total nitrogen content (table V), these same variations in sap insoluble nitrogen were evident except for the comparison between NaK_3 and K_1 in the mature leaves.

Sap soluble organic nitrogen was significantly lower in the mature leaves of NaK_3 plants than in those of the other treatments, when this fraction was expressed on the dry weight basis. In the expanding leaves NaK_3 was lower in this fraction than was K_1, K_2 or NaK_1. In addition, K_3 was lower than K_1 or NaK_1 in sap soluble organic nitrogen. However, when based on the total nitrogen, these variations in sap soluble organic nitrogen were not statistically significant. It may be noted that including the sap soluble organic nitrogen fraction with the sap insoluble fraction did not significantly alter the direction of the variations. Hence, variations in total organic nitrogen were essentially those of the sap insoluble fraction.

Amide nitrogen was significantly higher in the mature leaves of the K_1 plants than in those of other treatments. It might also be noted that the amide content of mature leaves was significantly lower in K_3 than in any of the series 2 treatments. Amides were lower in K_2 than in NaK_2. In the expanding leaves amides were significantly higher in K_1 than in any other treatment except NaK_1. These variations were common to both bases of expression.

Amino nitrogen was higher in the mature leaves of K_1 than in any treatment except NaK_2. In the latter treatment this fraction was higher than in K_2, K_3, or NaK_3. In the expanding leaves, the higher content of amino acids in K_1 than in any other treatment was the outstanding result. When expressed as percentage of the total nitrogen, amino acids in NaK_3 were higher than in K_2, K_3, or NaK_2.

Ammonium nitrogen was relatively low in all cases. In the mature leaves, this fraction was evidently higher in K_2 and K_3 than in K_1, NaK_1, or NaK_2. In the expanding leaves there was no significant variation. Nitrate nitrogen contents of mature leaves were significantly lower in the low potassium treatments than in intermediate or high potassium treatments of a given series. In the expanding leaves, K_3 and NaK_3 had higher nitrate contents than had other treatments.

Discussion

There can be no doubt that condensation of carbohydrates was restricted in the low potassium plants. In an effort to determine whether this effect was more closely related to the deficiency of potassium per se or to the accumulation of soluble calcium in these plants the ratio: levulins + inulin/reducing sugars has been employed as an index of condensation. This ratio does not strictly denote a definite proportion of combined to free fructose, since only a portion of the reducing sugar fraction is fructose (**42**).

Neither does it take into account variations in polymer size as between fractions of levulins and between levulins and inulin (41). However, this ratio may be used as an empirical index, if it is assumed that where polymerization of fructose is restricted, an accumulation of the reducing sugar fraction will result. Values for the ratio: levulins + inulin/reducing sugars obtained for leaf tissues were plotted against leaf tissue concentrations of potassium and calcium and against the several cation ratios. The best indication of a continuous relationship was found when the ratio: potassium/calcium was employed. In figure 1 are plotted treatment means for ma-

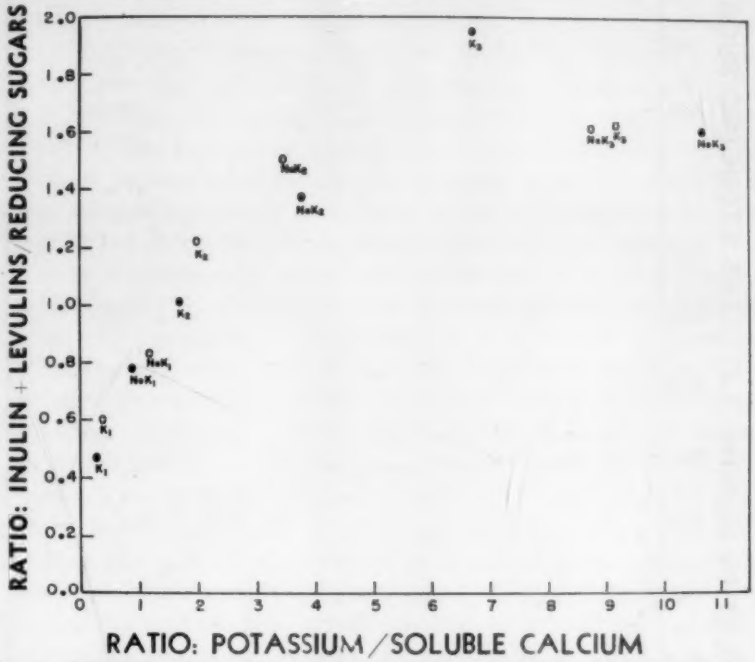

FIG. 1. Relationship between the ratio: m.e. K/m.e. soluble Ca and the ratio: levulins plus inulin/reducing sugars in mature and expanding leaves. Mean values for mature leaves (dots) and expanding leaves (circles) for all treatments are shown.

ture leaves and expanding leaves. No consistent departure from the general trend is associated with series or with tissue age groups. These plots appear to represent a trend concave to the axis defining the cation ratio. However, the curvilinear portion of the relationship seems confined to the range of excessive potassium. It is reasonable to suppose that in this range the condensation ratio has suffered secondary alteration resulting from reduced nitrogen assimilation (cf. nitrogen fractions) and reduced activity of the stem tip meristems (8).

Of greater interest is the range where condensation appears particularly limited by the cation ratio. This portion of the trend (Fig. 2) appears nearly linear. The correlation coefficient (+0.771) is relatively high

considering the variability between replicates for the carbohydrate fractions as indicated by the differences required for significance between treatment means (table II).

The data presented give good evidence of decreased polymerization of fructose to levulins and inulin in guayule plants deficient in potassium. This effect appears more closely related to the decreased ratio of potassium to soluble calcium than to the concentration of either element singly. It is of interest to recall that REED (**34**) found that in all green plants studied

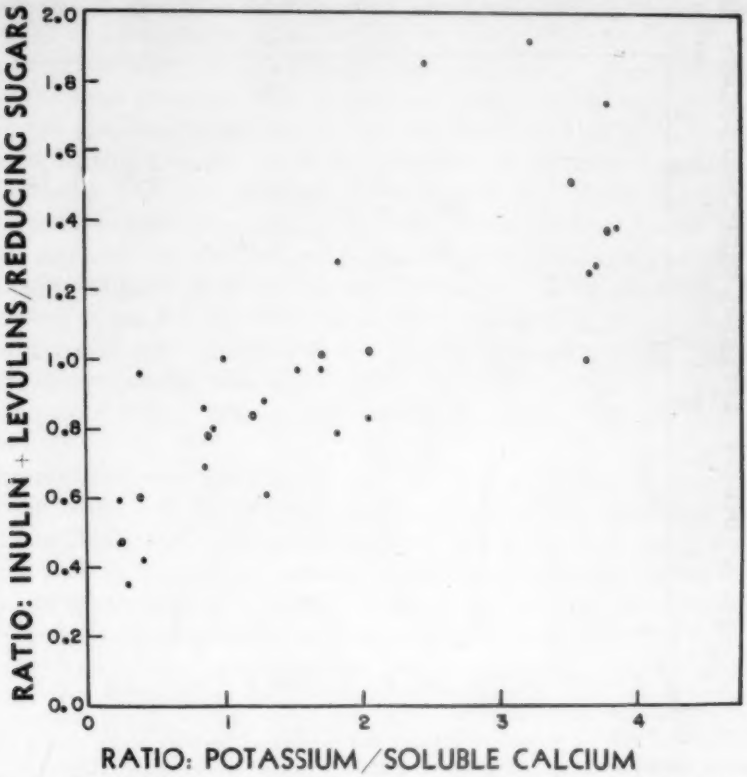

FIG. 2. Relationship between the ratio: m.e. K/m.e. soluble Ca and the ratio: levulins plus inulin/reducing sugars in mature and expanding leaves. Only low and intermediate potassium treatments are included. Dots represent individual samples. Circles denote treatment means for mature or expanding leaves. Coefficient of correlation = +0.771. Correlation coefficient required for significance = 0.515 ($n = 24$, $p = 0.01$).

by him, potassium was essential for starch formation. SIDERIS and YOUNG (**38**) in reviewing the literature pertaining to effects of potassium upon carbohydrates noted that plants supplied with small amounts of potassium contained in their leaves higher concentrations of reducing sugars than those with high potassium. High reducing sugar values in low potassium plants were usually accompanied by low values in disaccharides and polysaccharides, although exceptions were noted. This type of response was

distinguished from that in plants totally deficient in potassium where the amounts of reducing sugars were low (**14, 20**). SIDERIS and YOUNG obtained high values for reducing sugars and low values for sucrose and starch in low potassium pineapple plants. They noted that their data emphasize the necessity of adequate amounts of potassium for condensation of reducing sugars to sucrose and starch. CATTLE (**7**) floated detached leaves of normal and potassium deficient bean plants on glucose solutions in the dark. Greater synthesis of starch was found in normal leaves. KURSSANOV (**25**) cited data showing increased synthesis of sucrose from leaves infiltrated with potassium chloride. RUSSELL (**36**) found that in barley plants potassium deficiency resulted in low total sugar concentrations, but the concentrations of fructosan polysaccharides were decreased relatively more than were those of other sugars. These results were obtained in both high calcium and high sodium series. In connection with the present study the findings of Russell are of the greater interest since the fructosan of barley is characterized by linkage of carbon atoms 2 and 6, whereas the inulin linkage is between 1 and 2 positions (**1**). Of importance is the fact that similar effects upon polymerization of carbohydrates have been found to result from potassium deficiency in cases where the metabolism of the particular plant includes synthesis of fructosans of the cereal type, in plants where fructosans of the inulin type are formed, as well as those in which sucrose and starch are polymerization products.

The enzyme systems involved in synthesis of fructosans have not yet been isolated. However, as noted above, condensation of reducing sugars to sucrose and starch are also affected by potassium deficiency. It is known that starch is formed from glucose-l-phosphate with aid of a phosphorylase (**15, 16**). DOUDOROFF, KAPLAN and HASSID (**10**) have obtained from *Pseudomonas saccharophila* a preparation of a phosphorylase which catalyses the formation of sucrose from glucose-l-phosphate and fructose. Each of these reactions requires that phosphate first be transferred to the hexose molecule. The similarity between the mechanism of starch synthesis and breakdown in higher plants and that of glycogen in animal tissues and yeast has been pointed out by HASSID (**18**) and JAMES (**22**) who have recently reviewed the literature dealing with phosphorolysis in plants. According to current schemes of phosphate transfers in animal tissues high energy bond phosphate generated in the course of respiration is required for formation of hexose phosphates (**27**). Necessary for these transfers is the formation of adenosine triphosphate, which arises in part from the reaction: phosphopyruvate + adenosine diphosphate = pyruvate + adenosine triphosphate. LARDY and ZEIGLER (**26**) employing an enzyme preparation from rat muscle have shown this reaction to be reversible. The reaction is accelerated by potassium. Sodium could not replace potassium in this acceleration (**4**). Calcium was antagonistic to the action of potassium (**4, 5**). These findings suggest a scheme for the role of potassium in carbohydrate

condensation. If potassium can be shown to be necessary for this or similar reactions in plants, many of the conflicting views on the function of potassium can be rationalized, since the transfer of high energy phosphate is supposed to be a connecting link whereby energy released in respiration is transferred to various centers of anabolism.

In the low potassium treatments (K_1 and NaK_1) the expanding leaves had approximately normal contents of nitrate and total organic nitrogen. Total sap soluble organic nitrogen and insoluble nitrogen were not significantly altered by potassium deficiency in these leaves, although amides and amino acids accumulated in K_1. The results obtained for the mature leaves of the potassium deficient plants differ in several respects from those of mature leaves of the higher potassium treatments. The proportion of total organic nitrogen was higher than that of nitrate lower in the low potassium treatments. In addition the proportion of sap insoluble nitrogen was significantly higher in K_1 and NaK_1 than in other treatments. It is of interest that SIDERIS and YOUNG (**39**) have very recently reported similar results for leaves of *Ananas comosus*. Leaves of low potassium plants had lower nitrate, higher soluble organic nitrogen and higher protein concentrations than did high potassium plants. These authors attribute the lower nitrate concentrations in their low potassium plants to decreased nitrate absorption, since when expressed as milligrams per plant total nitrogen contents were lower in low potassium plants. However, the size of these plants was reduced. In the present experiment there was no reduction in absorption of nitrogen by the low potassium plants until after the size of these plants was reduced. Thus, although at final harvest the total milligrams of nitrogen absorbed by low potassium plants were less, the total weights of these plants were also less at this time. However, on February 12, when differences in weights were not yet apparent, low and intermediate potassium treatments resulted in absorption of practically identical quantities of nitrogen in each series (**4**). It appears that nitrate absorption was not directly effected by low solution concentrations of potassium.

RICHARDS and TEMPLEMAN (**35**) have emphasized the necessity of studying leaves of comparable "physiological age" when dealing with potassium deficiency. Thus where all "mature leaves" are composited, the result is to confound various stages of maturation and senescence. However, selection of criteria of physiological age proves very difficult where the metabolism of all stages is altered to the degree found in potassium deficiency. In the present case, it may be noted that the mature leaves of the potassium deficient plants were more similar to the expanding leaves with respect to proportions of nitrogen fractions than were the mature leaves of plants supplied with adequate potassium. Thus, the percentages of total nitrogen contributed by sap insoluble nitrogen, sap soluble nitrogen, total organic nitrogen, α-amino nitrogen, ammonium nitrogen, and nitrate nitrogen were similar in mature and expanding leaves in each of the treatments K_1 and NaK_1 (table V). The mature leaf samples obtained from K_1 plants were

on the average younger in actual age than mature leaves in K_2 plants inasmuch as a higher mortality of the oldest leaves had resulted in K_1. In NaK_1, the mortality of the old leaves was no greater than in NaK_2, so between these two treatments there was no great difference in actual age of the mature leaf samples. It therefore appears that in leaves of guayule plants deficient in potassium, one or more of the processes associated with maturation is altered.

The higher contents of α-amino and amide nitrogen in leaves of K_1 are in agreement with observations of some other investigators (**6, 17, 30, 39**). GREGORY (**13**) has held the view that such accumulation in the older leaves results from protein degradation, since these accumulations have sometimes been accompanied by lower protein contents. WALL (**45**) found that the ratio of soluble organic nitrogen to protein nitrogen was higher than normal in various parts of tomato plants which had been supplied with a solution lacking potassium. He concluded that potassium was necessary for synthesis of proteins from amino acids. SIDERIS and YOUNG (**39**) have found protein nitrogen contents of older leaves in pineapple plants supplied with low potassium to be equal to or greater than those from corresponding leaves of high potassium plants. The latter results are in agreement with the present findings. These authors have pointed to the fact that accumulations of soluble organic nitrogen fractions may be brought about by translocation of protein degradation products from dying tissues and parts to tissues still healthy in appearance. They, however, believe that no definite decision can be made as to whether these products are of synthetic or proteolytic origin. These arguments apply with equal force to the guayule leaves of this experiment. Thus, the K_1 plants in which amides and α-amino nitrogen accumulated had the greatest mortality of leaves which perhaps provided the greater supply of proteolytic products to leaves which were still living. On the other hand the nitrate content of the mature leaves of these plants was low, possibly indicating greater synthesis of soluble organic nitrogen fractions.

The high insoluble nitrogen contents of potassium deficient guayule leaves would not indicate that in these tissues protein synthesis was curtailed. However, it has been found by several workers (**6, 17, 39**) that although the protein content of potassium deficient leaves may be relatively high, the protein contents of the stems of these plants is frequently reduced. The protein concentrations in the guayule stems were not estimated, but it seems pertinent to note that growth in diameter of the low potassium stems was curtailed, whereas growth in length was not (**8**, table IV).

The conclusions of various investigators concerning the function of potassium in nitrogen metabolism are not in accord. Many of the differences in results obtained may arise from differences in degree of potassium deficiency, or from differences in the environmental conditions under which the experiments were conducted. In the present experiment with guayule

plants, potassium deficiency was not extreme, as indicated by the fact that only moderate reduction in growth of these plants resulted during the experimental period. The plants employed were relatively young. The experiment was conducted in the greenhouse during the winter months. It is quite possible that a different distribution of nitrogenous fractions would result in older plants, in other seasons, or where deficiency was more severe.

In the high potassium treatments (K_3 and NaK_3) expanding leaves had significantly less assimilated nitrogen and significantly more nitrate than expanding leaves of other treatments. These leaves, therefore, had not only a lower total nitrogen content, but in addition a less rapid rate of nitrate reduction. Although similar results were found in the mature leaves, these were not statistically significant. When it is considered that plants of these treatments were likely deficient in calcium at the time of final harvest (**8**), a lower rate of nitrate reduction conforms to the results of BURRELL (**6**). NIGHTINGALE *et al.* (**29**) noted low protein contents in calcium deficient plants and ascribed these results to a very limited ability of these plants to absorb nitrate and to restricted reduction of the nitrate absorbed. GAUCH (**12**) has since shown that the limited absorption of nitrate was associated with high magnesium contents of the minus calcium nutrient solutions employed. However, the restricted nitrate reduction is in agreement with the results reported here.

A relationship between nitrate reduction and malic acid accumulation has been suggested by other workers (**31**, **44**). When guayule leaves from the intermediate potassium treatments are used as a basis for comparison, high potassium leaves were relatively high in citric acid (**9**). Malic acid did not accumulate in high potassium leaves. This may indicate that transformation of citric acid to malic acid was inhibited in these leaves, since these acids are known to be interconvertible in some plant tissues (**43**, **22**). The tricarboxylic acid cycle of KREBS (**24**) indicates a possible course for oxidative formation of malic acid from citric acid. In minced breast muscle of pigeon dicarboxylic and tricarboxylic acids have been shown to catalyse oxidative respiration. In higher plants, such catalysis has not been demonstrated (**22**), but most of the organic acids and a number of the enzyme systems necessary for the reactions in Krebs cycle are known to occur (**2**, **3**, **22**). Whether or not these acids act as catalysts, it seems plausible that their oxidation in plants is brought about by a series of reactions similar to those of the tricarboxylic acid cycle. Partial inhibition of one of these reactions might result in accumulation of citrate as observed in guayule leaves from the high potassium treatments. A possible explanation for the accumulation of nitrate in these leaves is obtained, if it is further assumed that the reduction of nitrate is coupled with one of the oxidations in this series (i.e., dehydrogenation of isocitrate, α-ketoglutarate, or succinate) through the action of specific dehydrogenases and the appropriate phosphopyridine nucleotide.

Summary

1. The relative amounts of certain carbohydrate and nitrogenous fractions were determined in the tissues of guayule plants which had received low, intermediate, or high levels of potassium supply.

2. Reducing sugars in the leaves were consistently higher in low potassium treatments than in others. In old stems and roots levulins were consistently lower in low potassium than in intermediate potassium plants. In all tissues of the low potassium plants reducing sugars were high when expressed as percentage of the total carbohydrates. These results indicate that adequate potassium is required for condensation of fructose to fructosans of the inulin type in guayule. Condensation of fructose in the leaves of low potassium cultures appears more nearly related to the ratio: potassium/soluble calcium in the leaf tissues than to the tissue content of potassium per se. The significance of these results is discussed in relation to results from other plants where polysaccharides other than levulins and inulin are the principal products of hexose condensation.

3. Amide-nitrogen and α-amino nitrogen fractions were highest in mature and expanding leaves of the plants which contained the least potassium. Insoluble and sap soluble organic nitrogen fractions were at least as high in the leaves of potassium deficient plants as in those which had received an adequate potassium supply.

4. Leaves of high potassium plants contained less total organic nitrogen and more nitrate nitrogen than did plants of other treatments. The altered proportion of individual organic acids found in the high potassium leaves is considered in relation to limited nitrate reducton.

HAWAII AGRICULTURAL EXPERIMENT STATION
HONOLULU, HAWAII

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INVESTIGATIONS ON THE FLUOROMETRIC DETERMINATION OF MALIC AND SUCCINIC ACIDS IN APPLE TISSUE[1]

C. GUINN BARR[2]

(WITH THREE FIGURES)

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Introduction

The role which the organic acids play in plant metabolism is not well understood. The work of PUCHER, VICKERY, and WAKEMAN (**4, 5, 6, 7, 8, 9**) on tobacco and other plant materials has opened the way for many investigations on total organic acids and on the various individual organic acids such as malic, oxalic, pyruvic, citric, and succinic. In our investigations on the acid content of apples the need was felt for a rapid and reliable method for the estimation of mixtures of succinic and malic acid. While the existing methods for the determination of the total organic acid are relatively simple, the determination of succinic acid as succinyl p-toluide was not entirely satisfactory because of the difficulty of the processing of a large number of samples through all the steps of the method.

The fluorometric spot test described by FEIGL (**1**) for the estimation of dicarboxylic acids seemed promising for development into a rapid quantitative method. In this method the acids are heated with resorcinol and concentrated sulfuric acid and upon addition of alkali the fluorescence in ultraviolet light is measured. FEIGL (**1**) states that "dicarboxylic acids with the carboxyl groups in the 1,2 or 1,4 positions or their derivatives, such as esters, anhydrides, or imides, form dyes of the fluorescein type on melting with resorcinol," and these give a vivid greenish-yellow fluorescence in alkaline solutions by daylight and a greenish-blue fluorescence in ultraviolet light.

Under the conditions described by Feigl, malic acid forms semi-aldehydes of malonic acid or its homologues which condense with resorcinol to umbelliferone or its homologues, and these fluoresce a brilliant blue in alkaline solution under the mercury vapor lamp. It was found, however, that this solution is not stable in an alkaline reaction and the intensity of the fluorescence fades upon standing in aqueous solution at room temperature. PRINGSHEIM (**3**) states that in general any photoluminescent substance has an absorption band in the spectral region immediately adjoining the short-wave limit of the luminescence band and even somewhat overlapping it. Red, therefore, is excited by orange light, yellow, by green, green, by blue, and violet by ultraviolet.

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[2] Present address: John Bean, Division Food Machinery Corp., Lansing, Mich.

It was found that fluorescein shows a brilliant green fluorescence in alkaline solution if activated by the spectral region between 420 and 520 millimicrons isolated by Corning filters 3389 and 5551. The fluorescence is still more intense in acid solution (pH 1.5 to 2.0), a fact used to advantage in the measurement of the fluorescein derivative of succinic acid in the presence of the umbelliferone formed by malic acid, since umbelliferone does not fluoresce in acid solution. These facts seemed to offer a basis for determining these acids quantitatively.

A Pfaltz and Bauer Fluorophotometer was used throughout this work for measuring intensity of fluorescence. In the determination of succinic acid the instrument is calibrated with a standard solution of fluorescein. Using Corning filters No. 3389 and 5551 for transmission of the exciting light and Corning filters No. 3486 and 3387 as the secondary filter combination, the instrument is adjusted so that the galvanometer reads 100 per cent. for the fluorescence of a slightly acid solution containing 50 micrograms of fluorescein per 100 ml.

The blue fluorescence of umbelliferone is apparently of about the same wave length as the blue fluorescent light emitted by quinine sulfate when illuminated by ultraviolet light and a standard quinine sulfate solution can, therefore, be used for setting the instrument for quantitative determinations of malic acid. A Corning filter No. 5970 is used as the primary filter and for the secondary filters in the pick-up unit, Corning filters No. 4308 and 3389 are used. The fluorophotometer is adjusted to read 100 per cent. fluorescence for a solution containing 300 micrograms of quinine sulfate per 100 ml.

In order to obtain a straight line relationship between the concentration and the intensity of fluorescence of a substance, it is necessary to determine the proper range of concentration to use. Since the fluorescence of a substance is not proportional over all ranges of concentration, there is an upper limit to the concentration which can be used. Higher concentrations do not yield correspondingly greater fluorescence and the fluorescence may actually become less with increasing concentration. This behavior becomes very important when testing the fluorescence of samples of unknown concentration.

Determination of succinic acid

The detection and quantitative estimation of succinic acid has been carried out as follows: a stock solution containing 1 mg. of succinic acid per ml. is used to make the standard solutions for the subsequent calibration curve. For the preliminary work, three concentrations of 5, 10, and 15 mg. succinic acid per 100 ml. should be used. Two-ml. aliquots of these solutions are transferred to 5-ml. beakers or crucibles and evaporated to dryness in an oven at a temperature between 70° and 100° C. The beakers containing the succinic acid residue are cooled to room temperature and the dry succinic acid is moistened with 0.04 ml. of pure concentrated sul-

furic acid. This is stirred with a glass rod and about 10 mg. of freshly sublimed resorcinol are added and again stirred thoroughly to insure complete mixing of the resorcinol with the succinic acid-sulfuric acid mixture. The beakers are then placed in an oven at 126°–130° C. for one hour to allow the reaction to go to completion. The effect of time on the development of the fluorescence is discussed in another part of this paper.

When the reaction is complete, the samples are removed from the oven and cooled to room temperature. About 2 ml. of distilled water are added to dissolve the products. The solutions are transferred quantitatively to 200-ml. volumetric flasks, made to the mark with distilled water and mixed. Ten-ml. aliquots of these solutions are then transferred to 100-ml. volumetric flasks, 2 to 4 drops of (1 + 1) sulfuric acid are added to bring the pH to 1.5 to 2.0, and diluted to the mark. Portions of these solutions are used for the fluorescence tests. There are several steps in the procedure which are rather critical. No difficulty should be encountered, however, if a uniform technic is established. A reagent blank must be run with each set of determinations. The 5-ml. beakers containing the samples are conveniently handled by setting them in a small tray which can be placed in the oven. Twenty-five or thirty samples can be easily processed at a time in this way.

Discussion

It was found that the fluorescence of the succinic acid derivative may vary appreciably according to the order and manner of adding, and amount of reagents used. For example, it is essential that the dry succinic acid sample be moistened with 0.04 ml. of pure concentrated sulfuric acid before the resorcinol is added and the intensity of the fluorescence is decreased by the use of greater amounts of sulfuric acid. A quantity of resorcinol far in excess of the theoretical amount is required to insure complete reaction of all of the succinic acid present. The resorcinol should be freshly sublimed and powdered in an agate mortar and preliminary tests made to determine the requirements under the existing conditions. A small glass spatula or spoon can be made for dispensing the resorcinol. If a large excess of resorcinol is used, the reaction products may have a dark red color after treatment at 130° C., but the sensitivity of the tests is not altered. Neither is the sensitivity affected by the presence of malic acid in concentrations up to 15 micrograms per 100 ml. When a sample containing malic acid instead of distilled water is used as a reagent blank, a slightly higher fluorescence may be obtained for the blank reading but this is taken into account in calculating the net readings.

The succinic acid derivative is reasonably stable for several hours in the final dilute acidified solution. The intensity of the fluorescence will fade appreciably, however, if the samples are allowed to stand more than 48 hours. Fading has been observed also when the samples were allowed to remain longer than two hours in the 200-ml. volumetric flasks before aliquots were taken for fluorescence readings.

TABLE I

THE EFFECT OF VARYING QUANTITIES OF SULFURIC ACID UPON INTENSITY OF FLUORESCENCE

ML. OF SULFURIC ACID USED	INTENSITY OF FLUORESCENCE*	
	SUCCINIC ACID	MALIC ACID
0.02	89.0	100.0
	92.0	97.0
0.04	93.0	99.0
	96.0	98.0
0.06	96.0	100.0
	85.0	98.0
0.08	68.0	13.0
	78.0	26.0

* The values given are per cent. of light emitted as determined by use of the Fluorophotometer.

Crystallization of the resorcinol-sulfuric acid-organic acid mixture occurred before the samples were placed in the oven at 130° C. when more than 0.04 ml. of sulfuric acid was used with the quantity of resorcinol employed. In all cases the samples which did not crystallize fluoresced with greater intensity than those which crystallized.

Data are presented in table II which are typical of the results obtained repeatedly in testing the reliability of the succinic acid measurements in pure solution. These data are the results of six different sets of determinations with from two to five replications per set. Replications are individual samples carried through all the steps in the procedure and represent 2-ml. aliquots taken from standard solutions for evaporation.

Determination of malic acid

The procedure for the determination of malic acid is essentially the same as that used for succinic acid up to the step where the fluorescence is to be measured. Two-ml. aliquots of solutions containing 5, 10, and 15 mg. of malic acid per 100 ml. are evaporated to dryness and treated with

TABLE II

FLUORESCENCE INTENSITY OF THE FLUORESCEIN DERIVATIVE OF SUCCINIC ACID CONCENTRATION OF SUCCINIC ACID IN MICROGRAMS/100 ML.

5	10	15	5	10	15
29.5	61.5	87.5	31.0	62.0	84.0
32.0	62.0	86.0	32.0	62.0	87.0
33.0	56.0	86.0	25.5	58.5	85.5
33.0	54.0	84.0	28.5	53.5	84.5
32.0	56.0	87.0	29.5	60.5	85.5
33.0	56.0	87.0	33.0	63.0	91.0
31.5	56.5	83.5	33.0	63.0	92.0
29.5	57.5	80.5	31.0	58.0	85.0
27.5	62.5	84.5			
32.0	59.0	88.0	Av. 30.91	Av. 58.9	Av. 86.02

concentrated sulfuric acid and resorcinol, heated at 130° C. for 1 hour, transferred to 200-ml. volumetric flasks and made to the mark.

In the determination of the fluorescence of the malic acid derivative, 10-ml. aliquots of the solutions from the 200-ml. volumetric flasks are transferred to 100-ml. volumetric flasks and 1 ml. of 0.1 N NaOH are added before diluting to the mark. This brings the pH of the solution to about 10.5 at which maximum fluorescence is obtained. The fluorescence of these solutions must be measured without delay to avoid fading since the umbelliferone derivative is not stable at room temperature for more than an hour. Fluorescence intensities of the derivatives of succinic and malic acid in various concentrations are given in table III and the data are shown graphically in figure 1.

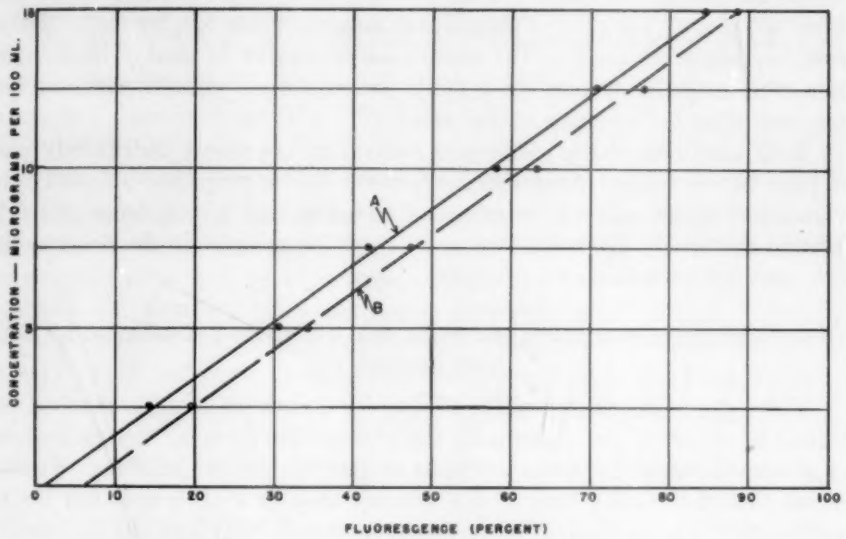

FIG. 1. The relation of succinic and malic acid concentration to the fluorescence of their resorcinol derivatives. (A) Succinic acid. (B) Malic acid.

DISCUSSION

At the outset it was found that alkaline solutions of the malic acid derivative appeared to be highly unstable. Fluorescence values were not uniform for replicate samples and the intensity decreased rapidly upon standing. If the samples were allowed to remain in a strongly alkaline reaction for 24 hours, the fluorescence dropped to about one-third of the original value and after two or three days the fluorescence was almost completely lost. The fluorescence could be restored and sometimes increased, however, by adding sulfuric acid to bring the pH to about 2 and again shifting the reaction back to pH 10 to 11 by the addition of sodium hydroxide solution. Further study showed that the rate of fading was somewhat dependent upon the concentration of the sodium hydroxide solution used. The addition of strong alkali (2 drops of 20 per cent. NaOH)

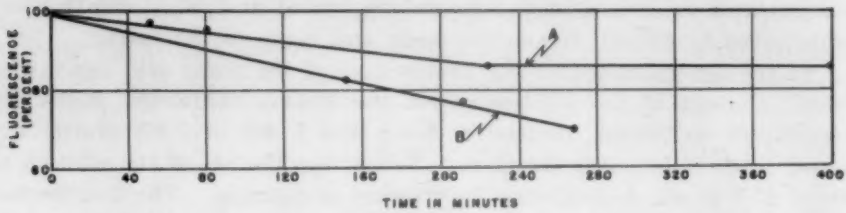

FIG. 2. Fading in fluorescence of the malic acid derivative after the addition of N alkali. (A) 1 ml. of 0.1 N NaOH. (B) 0.04 ml. of 20% NaOH.

caused a very rapid decay of fluorescence (a loss of about 30% in intensity after 4.5 hours). One ml. of 0.1 N sodium hydroxide added to the aliquot before making to volume, however, resulted in a loss of about 15 per cent. after 6.5 hours (Fig. 2). Fluorescence measurements can be made, therefore, on dilute solutions of the malic acid derivative in weakly basic reaction with minimum chance for error if the readings are made within a few minutes after the addition of the alkali.

Acid solutions of the malic acid derivative are stable indefinitely and it may be convenient, therefore, to pipette the appropriate aliquots into volumetric flasks, make to volume and mix, and add 2 to 4 drops of 0.1 N sodium hydroxide dropwise to a portion of the sample in the fluorometer cell just before making the reading.

Fluorescence of the succinic and malic acid derivatives as affected by time of heating

FEIGL (1) states that positive results for dicarboxylic acids may be obtained in the spot test technic by conversion into dyes of the fluorescein type upon heating the resorcinol melt at 130° C. for five minutes. It was found that the heating time of five minutes used in Feigl's spot test led to erratic results indicating an incomplete conversion in dyes of the fluorescein type. We determined that the maximum fluorescence is obtained after

TABLE III

THE EFFECT OF CONCENTRATION ON INTENSITY OF FLUORESCENCE

CONCENTRATION IN MICROGRAMS/100 ML.	INTENSITY OF FLUORESCENCE OF CONDENSATION PRODUCT	
	SUCCINIC ACID*	MALIC ACID†
2.5	14.0	19.3
5.0	30.7	34.3
7.5	41.9	47.5
10.0	58.5	63.3
12.5	71.0	76.8
15.0	84.9	89.0

* Fluorescein derivative of succinic acid, pH 1.5. Wave length of activating light between 420–520 millimicrons.

† Umbelliferone derivative of malic acid, pH 10.5. Wave length of activating light between 320–420 millimicrons.

TABLE IV

INTENSITY OF FLUORESCENCE AS AFFECTED BY TIME OF HEATING

MINUTES HEATED	INTENSITY OF FLUORESCENCE		
	SUCCINIC ACID		MALIC ACID
10 min.	60.5*	12.0†	83.5
20 min.	64.5	23.5	92.0
30 min.	85.0	24.0	93.0
45 min.	86.5	47.4	97.0
60 min.	98.5	79.0	97.0
75 min.	90.5	76.7	93.5
90 min.	92.0	77.5	86.5
105 min.	95.0	78.7	87.5
120 min.	92.0	79.3	89.5

* Heated with 0.04 ml. of sulfuric acid. † Heated with 0.06 ml. of sulfuric acid.

heating the mixture for one hour at 130° C. The results of these experiments are assembled in table IV.

Detection of succinic and malic acids in pure and in mixed solutions

Using solutions of known concentration, a calibration curve, for use in the estimation of unknown amounts of succinic and malic acid, should be constructed with each set of determinations. In quantitative fluorometric analysis, the accuracy of measurement decreases as the values approach either extreme of the galvanometer scale. Discrepancies, therefore, are to be expected in detecting organic acids in concentrations less than 5 micro-

TABLE V

DETERMINATION OF SUCCINIC ACID IN MIXED SOLUTIONS OF MALIC AND SUCCINIC ACIDS

AMT. OF ORGANIC ACID PRESENT (MICROGRAMS/100 ML.)		NET FLUORESCENCE	MICROGRAMS OF SUCCINIC ACID DETERMINED	VARIATION FROM THEORETICAL (MICROGRAMS)
SUCCINIC	MALIC			
0.0	5.0	0.0	0.0	.....
1.0	5.0	6.1	1.0	0.0
2.0	5.0	10.0	1.7	−0.3
4.0	5.0	21.0	3.6	−0.4
5.0	5.0	24.5	4.2	−0.8
0.0	10.0	0.0	0.0	.....
1.0	10.0	5.0	0.8	−0.2
2.0	10.0	9.0	1.5	−0.5
4.0	10.0	24.0	4.1	+0.1
5.0	10.0	31.0	5.3	+0.3
10.0	10.0	55.0	9.4	−0.6
0.0	15.0	0.0	0.0	.....
1.0	15.0	8.3	1.4	+0.4
2.0	15.0	14.0	2.4	+0.4
4.0	15.0	25.0	4.2	+0.2
5.0	15.0	31.0	5.3	+0.3
10.0	15.0	59.0	10.0	0.0
15.0	15.0	87.0	15.0	0.0

grams per 100 ml. when the instrument is calibrated for detection of quantities ranging between 5 and 15 micrograms per 100 ml.

Various amounts of succinic acid were added to three different concentrations of malic acid. Results of the succinic acid determinations are shown in table V. Estimations of succinic acid within one microgram per 100 ml. were made and apparently the presence of malic acid in the proportions used had no important effect on the accuracy of the determinations.

Malic acid in pure solution can be determined with reasonable accuracy. The presence of succinic acid and other organic acids that yield substances which show a bluish-green fluorescence under ultraviolet light after treatment with resorcinol and sulfuric acid increase slightly the apparent malic acid values. Some of this fluorescence is screened out, however, by the secondary filters. The fluorescence of the succinic acid derivative, furthermore, is quenched materially in the presence of comparable concentrations of umbelliferone. In this connection it should be pointed out also that the fluorescence of the fluorescein, produced by small amounts of succinic acid which may be present, is not effectively excited by the relatively low light requirements of the activating beam used for malic acid determinations (table VI).

TABLE VI

THE EFFECT OF VARYING QUANTITIES OF SUCCINIC ACID UPON THE INTENSITY OF FLUORESCENCE OF MALIC ACID DERIVATIVES

AMT. OF ACID PRESENT (MICROGRAMS/100 ML.)		INTENSITY OF FLUORESCENCE FOR MALIC ACID (NET)
MALIC	SUCCINIC	
5	0	35.5
5	1	32.0
5	2	34.5
5	4	30.0
5	5	34.0
5	10	35.0
5	15	31.0
10	0	62.5
10	1	65.0
10	2	61.5
10	4	65.0
10	5	64.5
10	10	61.0
10	15	64.0
15	0	88.5
15	1	88.0
15	2	89.0
15	4	90.0
15	5	89.0
15	10	88.0
15	15	91.0

Results of studies on the recovery of malic acid in the presence of varying amounts of succinic acid are presented in table VII.

TABLE VII

DETERMINATION OF MALIC ACID IN MIXED SOLUTIONS OF SUCCINIC AND MALIC ACIDS

AMT. OF ORGANIC ACID PRESENT (MICROGRAMS/100 ML.)		NET FLUORESCENCE	MICROGRAMS OF MALIC ACID DETERMINED	VARIATION FROM THEORETICAL (MICROGRAMS)
MALIC	SUCCINIC			
5.0	0.0	0.0	.....	.....
5.0	2.0	16.4	2.6	+0.6
5.0	4.0	24.4	3.9	−0.1
5.0	5.0	30.9	4.9	−0.1
10.0	0.0	0.0	.....	.....
10.0	2.0	18.0	2.8	+0.8
10.0	4.0	23.0	3.7	−0.3
10.0	5.0	27.6	4.4	−0.6
10.0	10.0	59.0	9.4	−0.6
15.0	0.0	0.0	.....	.....
15.0	2.0	15.0	2.4	+0.4
15.0	4.0	25.0	4.0	0.0
15.0	5.0	31.5	5.0	0.0
15.0	10.0	65.3	10.4	+0.4
15.0	15.0	88.0	14.1	−0.9

Detection of succinic and malic acids in plant extracts

A method for determining succinic and malic acids in pure solutions and in solutions of mixed pure organic acids to be of value must be suitable for the analysis of extracts of biological tissue. The organic acid fraction was extracted from apple tissue by a method essentially as described by ISAACS and BROYER (**2**). Aliquots of 2, 5, and 10 ml. of uniform samples of the extract were diluted to 100 ml. and designated as samples A, B, and C. Determinations of malic acid were made by the fluorometric method described above and compared with the results obtained by the oxidation method of Pucher and Vickery. The results are shown in table VIII.

Results of the fluorometric method of analysis for the determination of succinic and malic acid in apple tissue extracts containing known additional amounts of these acids are presented in table IX. Our studies

TABLE VIII

ESTIMATION OF MALIC ACID IN APPLE TISSUE EXTRACT

SAMPLE	MALIC ACID CONTENT IN MG./ML. OF ORGANIC ACID EXTRACT*	
	FLUOROMETRIC METHOD	OXIDATION METHOD
A	1.80	1.90
B	2.16	2.00
C	2.56	2.45

* Each ml. organic acid extract represents 2.00 gm. fresh apple tissue.

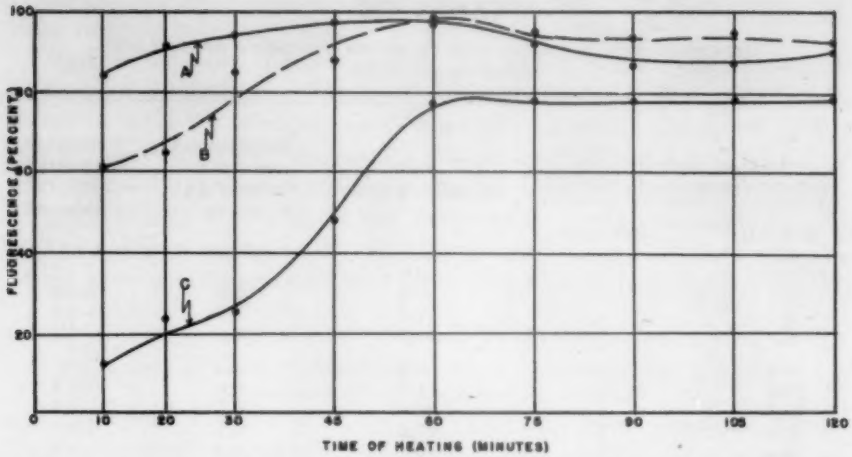

FIG. 3. Intensity of fluorescence as affected by time of heating. (A) Malic acid—using 0.04 ml. of sulfuric acid in the reaction. (B) Succinic acid—using 0.04 ml. of sulfuric acid in the reaction. (C) Succinic acid—using 0.06 ml. of sulfuric acid in the reaction.

showed the estimations for malic acid can be made with about the same degree of accuracy as those for succinic acid. Most accurate results are obtained when the fluorometric readings are between 30 and 80 on the scale of the galvanometer.

Determinations were made by the fluorometric method of succinic and malic acid in tobacco and corn leaf-tissue. The succinic acid content of

TABLE IX

DETERMINATION OF SUCCINIC AND MALIC ACIDS IN EXTRACTS OF APPLE TISSUE

NET FLUORESCENCE	MICROGRAMS/100 ML.				VARIATION FROM THEORETICAL (MICROGRAMS)
	PRESENT	ADDED	TOTAL PRESENT	DETERMINED	
	SUCCINIC ACID				
7.0	1.0	0.0	1.0	1.2	+0.2
17.0	1.0	2.0	3.0	2.9	−0.1
30.0	1.0	4.0	5.0	5.1	0.0
34.5	1.0	5.0	6.0	5.8	−0.2
60.3	1.0	10.0	11.0	10.2	−0.8
88.0	1.0	15.0	16.0	15.0	−1.0
	MALIC ACID MICROGRAMS /100 ML.				
35.8	5.0	0.0	5.0	5.4	+0.4
43.5	5.0	2.0	7.0	6.9	−0.1
47.0	5.0	2.5	7.5	7.5	0.0
61.5	5.0	5.0	10.0	9.8	−0.2
74.0	5.0	7.5	12.5	12.1	−0.4

tobacco leaves was 0.10 per cent. and that of corn 0.05 per cent. (fresh weight basis); whereas, the acid content estimated as malic was 1.02 and 0.15 per cent. (fresh weight basis) for tobacco and corn leaves, respectively.

The malic acid content of plant tissue varies materially with the species and with the part of the plant studied. Leaf tissue, in general, is relatively low in malic acid content, while fruits, especially apples, usually contain appreciable quantities. Furthermore, succinic acid normally constitutes only a small percentage of the total organic acid content and the presence of naturally occurring succinic acid will ordinarily have practically no effect on the malic acid determinations. The malic acid derivative shows virtually no fluorescence in acid reaction when measurements are made for succinic acid and relatively high concentrations of malic acid may be present without interference.

The presence of citric acid and other substances which yield compounds of the umbelliferone type when treated with resorcinol and sulfuric acid is troublesome in evaluating the malic acid content. Preliminary investigations have shown a direct relationship between fluorescence and concentrations of citric acid in terms of the resorcinol derivative. The fluorometric method is suitable, however, for malic acid when only malic, or malic and succinic are present. In the presence of citric acid the total amount of both malic and citric may be determined and the malic acid content calculated by difference after determining the percentage of citric acid by some other means. The author has found the fluorometric method as described herein to be reliable for the determination of succinic acid. Although the fluorometric procedure was developed primarily for the determination of succinic and malic acid in apple tissue, results obtained in this investigation indicate that the method should be adaptable to other tissues.

Summary

Succinic acid and malic acid when heated with resorcinol and sulfuric acid yield compounds which show intense fluorescence under specific conditions. Fluorometric separation of the succinic- and malic-acid derivatives has been accomplished by controlling the exciting light and the reaction of the test solution. Dilute solutions of the succinic acid derivatives show an intense green fluorescence in acid reaction when illuminated by light of wave length 420 to 520 millimicrons. The malic acid derivative shows virtually no fluorescence under these conditions but fluoresces a brilliant blue in basic solution under ultraviolet light of 320 to 420 millimicrons in length.

A photometric method based on these properties has been developed for the quantitative determination of these acids in mixed solutions, and in certain plant tissues.

The author expresses his thanks to Dr. Erwin J. Benne of the Agricultural Chemistry Department for many helpful suggestions in the prepara-

tion of the manuscript. Kind thanks are also due Miss Selma Bandemer for her counsel during the early stages of this investigation.

MICHIGAN AGRICULTURAL EXPERIMENT STATION
EAST LANSING, MICHIGAN

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THE ABSORPTION AND FLUORESCENCE SPECTRA OF TWO RED MARINE ALGAE

RICHARD W. VAN NORMAN, C. S. FRENCH,[1] AND FERGUS D. H. MACDOWALL[1]

(WITH FIVE FIGURES)

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Introduction

The problem as to whether or not there are pigments other than chlorophyll which can participate directly in photosynthesis by acting as photocatalysts has been discussed for many years. One of the earliest pieces of quantitative evidence for this possibility was the work of Warburg and Negelein which showed that the light absorbed by carotinoids was probably at least partially active in photosynthesis. Definite evidence for the participation of the carotinoid, fucoxanthin, as a light absorbing pigment for photosynthesis was provided by DUTTON and MANNING (**1**). It was later shown by DUTTON, MANNING and DUGGAR (**2**) that the light absorbed by fucoxanthin was in some way transferred to chlorophyll rather than itself acting directly upon the remainder of the photosynthesis mechanism. This was established by showing that the fluorescence of chlorophyll was excited equally well by red light which is primarily absorbed by chlorophyll and by blue-green light which is primarily absorbed by fucoxanthin. Phycocyanin was found by EMERSON and LEWIS (**4**) to be a photosynthetic pigment. It has not yet been established whether phycocyanin acts directly in the same way as does chlorophyll, or whether phycocyanin like fucoxanthin first transfers its energy to chlorophyll. Recently, HAXO and BLINKS (**8**) have reported that green light which is absorbed largely by the pigment phycoerythrin is several times as effective as is red light in photosynthesis of several red marine algae. The question arises as to whether the extra photosynthesis given by green light is due to a stronger absorption of light in this region by the algae or if this greater effectiveness of green light is due specifically to greater efficiency of phycoerythrin in photosynthesis. If the latter is the case, is phycoerythrin a pigment which acts by transferring absorbed light to chlorophyll or does it take part directly in the photosynthetic mechanism without the intermediate assistance of chlorophyll? Our measurements show only slightly greater absorption of green than of red light in two species of red algae. We have studied various aspects of the absorption and fluorescence of the pigments of red marine algae which are fundamental to the investigation of the question of the means by which phycoerythrin participates in photosynthesis.

[1] Present address: Carnegie Institution of Washington, Division of Plant Biology, Stanford, California.

Results

Materials

Two batches of algae were available to us for these experiments. The first batch was collected by one of us (F.D.H.M.) near Victoria, British Columbia, and brought to the laboratory by airplane. The second batch was collected in the same place and shipped by air express in two one-gallon thermos jugs. The temperature of this shipment on arrival was 17° C. Several species of algae from each batch were tested for their capacity to perform photosynthesis. In the first batch *Gigartina radula, Iridaea sp.,* and Ulva were found to give no photosynthesis. There was no gas evolution when this Gigartina was tested for O_2 evolution by the Hill reaction while being illuminated in a solution of quinone or in a mixture of ferric oxalate and ferricyanide. From these experiments it appears unlikely that any of the algae in the first batch were photosynthetically active at the time the fluorescence experiments were performed with them. In the second batch of algae *Gigartina exasperata* and *Iridaea* showed photosynthesis rates measured manometrically in sea water of about half of those reported by Emerson and Green (**3**) for a species of Gigartina. Our lower rate may have been due to the use of a somewhat lower light intensity, which in our case was 250 f.c. The *Gigartina radula* appeared to be the least stable of all the algae since some of its red pigment diffused out into the sea water during transit. It appeared to be much more fragile than the other species with which we were dealing and did not show any photosynthesis.

The absorption spectra of several intact algae

The absorption spectra of several of the algae were measured with the Ulbricht sphere and monochromator which has been described previously (**5, 11**). The algae wet with sea water were placed in the bottom of Carrel culture flasks which were held in the center of the sphere. The correction for reflected and absorbed light in the pyrex vessel was applied as previously described (**11**). Our results are presented in figure 1 in which the light absorption in per cent. is plotted as a function of wavelength. Band widths of about 14 mμ were used and stray light was eliminated by use of the filters listed in (**11**).

At about 710 mμ the absorption of the two red algae is somewhat above that of the brown alga Alaria, although Alaria absorbs much more strongly at the chlorophyll-a maximum. The slightly greater absorption at 710 mμ may perhaps be due to the presence of chlorophyll-d. The chlorophyll-a maximum appears at about 675 mμ. The maximum obtained at 620 mμ we attribute to the presence of phycoerythrin. It is also likely that the minor absorption band of chlorophyll-a contributes to the increase in absorption at 620 mμ. The minimum absorption occurs at 600 mμ in sharp contrast to the behavior of green leaves which all show a minimum at 550 mμ. From this point on, the curve rises to a broad maximum at

about 550 mμ. In Gigartina, there appears to be a slight depression and another rise at 530 mμ. This rise has not been identified with any particular pigment. In Iridaea there is but one very broad band with the maximum at about 550 mμ. The maximum found at 495 mμ is a band also present in green leaves and may be presumed to be due either to a chlorophyll or to a carotinoid. However, we see in figure 2 below that the water extract of Iridaea containing primarily phycoerythrin also shows a sharp maximum at this point. It may be that the absorption spectrum of the intact algae rises in region of 495 mμ because of the combined effects of several pigments including phycoerythrin. From 470 mμ on toward the shorter wavelengths

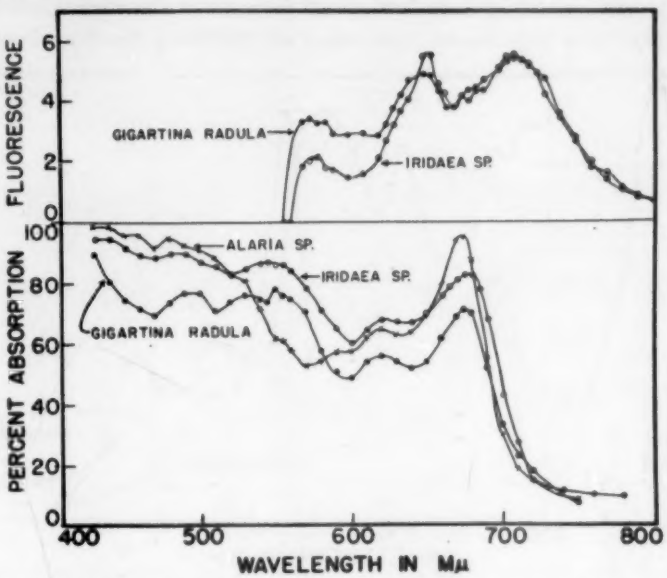

FIG. 1. The absorption spectra of two red and one brown marine algae, with the fluorescence spectra of the two red algae plotted above.

the curves rise rather sharply. This also is noted in both the methanol-soluble and the water-soluble pigments which will be discussed later. In the curve for Iridaea the bands are much less pronounced, but the maxima and minima are found in the same positions as in the curve for *Gigartina radula*. A point worthy of note is that the absorption of *Gigartina* at 550 mμ is 78% while the absorption at the red maximum, in this case 675 mμ is 71%. If the algae used by Haxo and Blinks are even approximately similar, their several times higher yield of photosynthesis in green light can not have been due to increased absorption and therefore must be due to participation of phycoerythrin in the process of photosynthesis. In comparing the height of the absorption band at 550 mμ in Gigartina with that of Lactuca (**11**), we conclude that the absorption due to phycoerythrin in *Gigartina radula* is about three times that of the absorption due to chlorophyll at this wavelength.

The curve for Alaria, a brown alga, is much more like that of a typical green leaf. The red maximum appears to be at 675 mμ with a minimum at 570 mμ. From this point on toward the blue the rise is presumed to be primarily due to absorption by fucoxanthin.

A PARTIAL SEPARATION OF THE PIGMENTS OF IRIDAEA IN EXTRACTS

In order to study both the absorption and the fluorescence of phycoerythrin and of chlorophyll for the most part separated from each other, extracts were made from a fresh piece of Iridaea. This was done by grinding with sand and fine quartz powder with added water. This macerated material was centrifuged at low speed to remove the larger particles and then placed in a high speed centrifuge at 12,000 × g for 20 minutes. Two

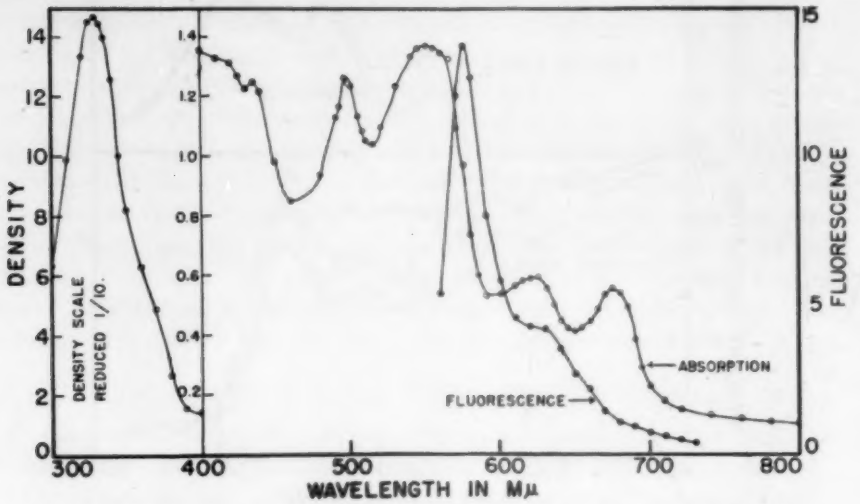

FIG. 2. The absorption and fluorescence spectra of a water extract of *Iridaea sp.*, containing primarily phycoerythrin.

successive water extracts were combined for the high speed centrifugation. The supernatant material will be referred to hereafter as the water extract of Iridaea. To the residue of the water extraction a volume of methyl alcohol was added which was equal to the total volume of cell material and quartz. The first methyl alcohol extract was slightly pinkish but contained no appreciable amount of pigment and was rejected. The second extraction with an equal volume of methyl alcohol was green in color. This, combined with a third methyl alcohol extract which was deep green was saved and will be referred to later as the methanol extract of Iridaea. A fourth extraction with methanol, made by heating the mixture to boiling, was brownish presumably due to the formation of some phaeophytin. This fraction was rejected.

The water extract was pink and highly fluorescent. It was nearly clear but did show some cloudiness which was hard to distinguish due to the

intense orange fluorescence. Its absorption curve is shown in figure 2. The absorption maximum at 675 mμ indicated the presence of some chloroplastin. The absorption due to chlorophyll present compared to that of phycoerythrin appeared to be quite small. Maximum of phycoerythrin absorption appeared at a wavelength of 550 mμ. It is a rather broad band extending from 540 to 570 mμ. We did not find a minimum at 550 mμ between two peaks as described by KYLIN (10). This band is followed by a minimum at 515 mμ and another maximum at 495 mμ. A broad minimum at 460 mμ leads to a steep rise at the blue end of the spectrum. In the near ultraviolet a very dense band was found with a maximum at 329 mμ. This absorption was ten times that of any of the visible part of the spectrum. Since this was a crude extract of the algae and not a

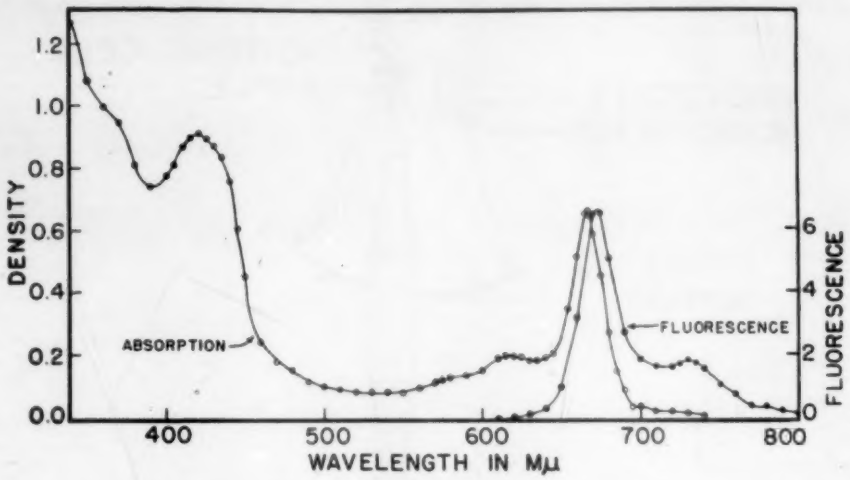

FIG. 3. The absorption and fluorescence spectra of a methanol extract of *Iridaea sp.*, containing primarily chlorophylls and carotinoids.

purified preparation it remains to be determined if this band is due to phycoerythrin.

The absorption curve of the methanol extract of Iridaea is shown in figure 3. It was hoped that this extract might reveal some trace of the chlorophyll-d band in the neighborhood of 690 mμ. It is, however, difficult to tell whether or not chlorophyll-d is present in traces in this extract. The chlorophyll-a maximum is found to be at 666 mμ. HARRIS and ZSCHEILE (9) report a major maximum for chlorophyll in methanol at 664 mμ.

We have attempted to reproduce the absorption curve of the algae by the addition of curves of the water extract and of the methyl alcohol extract. To make such an addition, an appreciable broadening of the chlorophyll band is required in addition to the shifting of its maximum. This appears to be reasonable in that the pigment combined with the protein may well have

a broader band than a pigment in true solution. However, any procedure which we were able to devise for broadening this red band resulted in curves which did not fit at the blue end of the spectrum so the attempt to reproduce the intact algae curves by some manipulation of the extract curves was abandoned. We did not, however, find any definite evidence for the presence of other pigments than those accounted for in one or another of these extracts. A possible exception to this statement may be the small maximum found at 530 mμ in the absorption of *Gigartina radula*.

The intensity of red fluorescence emitted by intact algae when excited by equal numbers of quanta of various wavelengths

We have tried to find out if the energy which is absorbed by phycoerythrin is transferred to chlorophyll. If this were the case one should be

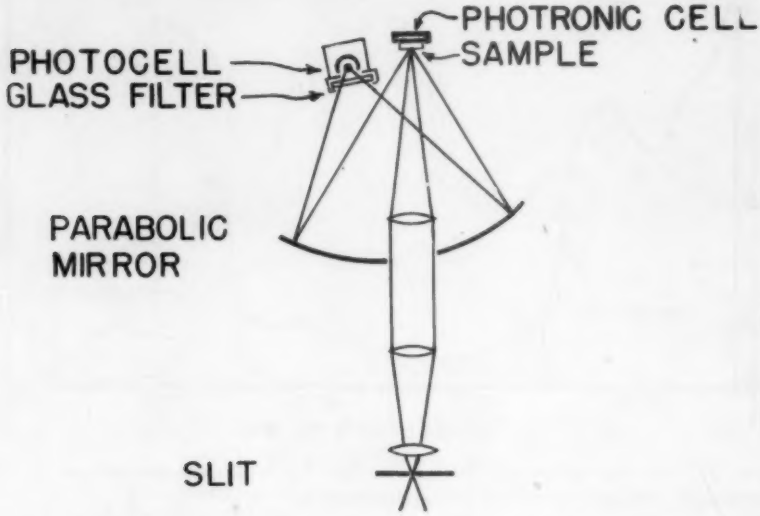

Fig. 4. The apparatus used to measure the intensity of red fluorescence emitted by intact algae when excited by equal numbers of quanta of various wavelengths.

able to get high chlorophyll fluorescence from the algae when they are absorbing light which is primarily taken up by phycoerythrin. If the red fluorescence of the algae were due only to chlorophyll the question could be settled by measuring the fluorescence passing through a red filter. This approach has been used successfully by Dutton, Manning and Duggar (**2**) in the study of light absorption by fucoxanthin. We were not able to answer this question satisfactorily in the case of phycoerythrin. We have, however, obtained considerable information which should help in the eventual solution of this problem.

Measurements of the relative intensity of fluorescence with different exciting wavelengths were made with the apparatus diagrammed in figure 4. A sample was held in a Carrel culture flask and illuminated by a beam of

TABLE I

A SAMPLE SET OF DATA AND CALCULATIONS OF THE RELATIVE FLUORESCENCE YIELDS IN AN INTACT ALGA EXCITED BY DIFFERENT WAVELENGTHS

	INCIDENT ENERGY MEASUREMENTS	
Wavelength:	560 mμ	650 mμ
Band width: Stray light filters:	14 mμ (2nd order; 2-mm. slits) 2.6 cm. $FeSO_4$ sat. in 25% H_2SO_4 2.6 cm. $CuSO_4$ dilute soln. Corning Nos. 3484, 4305, 512	14 mμ (1st order; 1-mm. slits) 2.6 cm. $FeSO_4$ sat. in 25% H_2SO_4 2.6 cm. $CuSO_4$ dilute soln. Corning No. 241
Total of thermopile deflections at 1-mm. intervals across the beam $\times$ 1/width of receivers:	6.75 cm.	3.56 cm.
Defln. of thermopile exposed to standard lamp giving 2.35×10^{-3} cals./min. at 1 M.:	15.5 cm.	
Energy incident on algae:	$\frac{6.75}{15.5} \times 3.35 \times 10^{-3} =$ 1.46×10^{-3} cals./min.	$\frac{3.56}{15.5} \times 3.35 \times 10^{-3} =$ 7.7×10^{-4} cals./min.
Energy of one quantum:	8.39×10^{-20} cals./min.	7.22×10^{-20} cals./min.
Correction for Pyrex cell transmission:	0.91	
Energy incident on algae:	$0.91 \times \frac{1.46 \times 10^{-3}}{8.39 \times 10^{-20}} =$ 1.55×10^{16} quanta/min.	$0.91 \times \frac{7.7 \times 10^{-4}}{7.22 \times 10^{-20}} =$ 0.97×10^{16} quanta/min.
	FLUORESCENCE MEASUREMENTS	
Photronic cell galv. defl. with no sample:	51.0 cm.	13.3 cm.
Photronic cell galv. defl. with algae:	7.0 cm.	4.3 cm.
Fraction absorbed:	0.86	0.68
Energy absorbed:	1.33×10^{16} quanta/min.	6.59×10^{15} quanta/min.
Photocell response with MgO: Filter before photocell:		
(RG8)	0	7.2
(88)	0	4.0
(5850 + 241)	0	4.0
Correction for reflection:		
(RG8)	0	$0.2 \times 7.2 = 1.4$
(88)	0	$0.2 \times 4.0 = 0.8$
(5850 + 241)	0	$0.2 \times 4.0 = 0.8$
Deflection from algae:		
(RG8)	43.0	18.0
(88)	32.0	13.8
(5850 + 241)	31.5	11.5
Deflection corrected for reflection:		
(RG8)	43.0	16.6
(88)	32.0	13.0
(5850 + 241)	31.5	10.7
Fluorescence per 10^{15} absorbed quanta:		
(RG8)	$\frac{43.0}{13.3} = 3.23$	$\frac{16.6}{6.59} = 2.52$
(88)	$\frac{32.0}{13.3} = 2.40$	$\frac{13.0}{6.59} = 1.97$
(5850 + 241)	$\frac{31.5}{13.3} = 2.36$	$\frac{10.7}{6.59} = 1.62$

light of about two sq. cm. area which came from the monochromator. Filters (11) were used in front of the monochromator to reduce stray light of other wavelengths. The intensity of the incident light was measured by a thermopile calibrated in absolute units. The fraction of light which was absorbed by the algae was measured by a photronic cell placed directly behind the vessel. Its diffuse reflection was estimated as a fraction of incident light, by coating half the vessel with magnesium oxide and weakening that portion of the beam of light which fell on the magnesium oxide with a set of filters of known transmission until the brightness of the magnesium oxide and of the algae appeared equal. This value was used in making a correction for scattered light as noted below. The fluorescent light was collected by means of a curved mirror which concentrated a constant fraction thereof on a photocell located behind red glass filters. The photocell was connected to a "Photovolt" amplifier which permitted reading

TABLE II

THE RATIOS OF FLUORESCENCE YIELD FROM RED AND FROM GREEN EXCITING LIGHT ON THE BASIS OF EQUAL ABSORBED QUANTA. THIS SHOWS THAT MORE RED FLUORESCENCE IS OBTAINED FROM LIGHT ABSORBED PRIMARILY BY PHYCOERYTHRIN THAN FROM LIGHT ABSORBED BY CHLOROPHYLL

SPECIES	FILTER			
	RG8	88	5850 + 241	AVERAGE
	Fl. from 560 mμ / Fl. from 650 mμ			
Gigartina radula	.....	.....	1.9	1.9
" "	2.1	2.1	1.8	2.0
" "	1.7	4.3	1.3	2.6
Iridaea sp.	.....	.....	1.2	1.2
"	1.3	1.2	1.5	1.3
Bean leaf	0.66	0.79	0.70	0.72
Magnolia acuminata	0.67	0.80	0.61	0.69

of the relative intensity on the photocell as a galvanometer deflection. When magnesium oxide was substituted for the sample there was a measureable galvanometer deflection. The scattered light was corrected for by multiplying the reflection of a sample by the galvanometer deflection introduced by the magnesium oxide, and subtracting this number from the apparent fluorescence of the sample.

Three different sets of filters were used between the fluorescent algae and the photocell: Corning ♯5850 with ♯241, which cuts off 50% of the light at about 720 mμ; Wratten ♯88, which has a 50% transmission at about 710 mμ; and Jena RG8, which transmits 50% of the light at about 703 mμ. A list of sample calculations for a complete set of measurements is presented in table I. The results of all such measurements are presented as ratios in table II. No attempts were made to measure the percentage yield of the incident light which was turned into fluorescent light.

It was observed (table II) that in the red algae the fluorescence excited by green light having a wavelength of 560 mμ is definitely larger than that excited by red light having a wavelength of 650 mμ. The green light is largely absorbed by phycoerythrin and the red by chlorophyll. In comparison with this behavior of red algae it is seen that in green leaves the relative fluorescence yields are reversed; that is, more red fluorescence is excited by red light than by green light. These results could be due either to a red fluorescence from other pigments than chlorophyll or to a more efficient excitation of chlorophyll fluorescence by phycoerythrin than by chlorophyll itself. A study of the fluorescence spectra of the live algae and of the extracts was therefore undertaken.

The fluorescence spectra of intact algae and of their extracts

The algae in a glass vessel containing sea water were exposed to the green and blue lines of the mercury arc obtained from an H-4 mercury lamp filtered through a double Corning ♯9780 filter and also a Corning #512 thus transmitting the 365, 405, 436 and 546 mμ lines. An image of the mercury lamp fell on the surface of the algae at an angle of incidence of about 70°. The fluorescent light was collected by a short-focus lens and focused on the slit of the monochromator. Behind the entrance slit was placed an orange Corning ♯3842 filter to absorb the blue light completely and a very large fraction of the green light. Measurements were made at every 10 mμ over the range from 560 to 800 mμ of the intensity of fluorescence with a photocell which was placed behind the exit-slit of the monochromator. At each wavelength the deflection of the photocell was compared with the deflection produced when light from a standard lamp of known energy distribution was allowed to go through the same optical system. Its intensity was reduced by means of wire screens so that it was of a magnitude comparable with the intensity of the fluorescent light from the algae. In this way the relative energy distribution throughout the spectrum of the fluorescent light could be determined and the photocell sensitivity and monochromator transmission were thus cancelled out. The curves which were obtained using the apparatus shown in figure 5 have been plotted in figure 1 to show their position in relation to the absorption of the algae. It will be noted that the fluorescence of phycoerythrin which had its maximum at about 575 mμ is small in proportion to the height of the fluorescence bands in the red region. There are two red maxima in the fluorescence spectra of both algae investigated and we have so far been unable to attribute these bands definitely to any particular pigment. It is striking, however, that the minimum between these two bands comes at exactly the same place as the maximum of the chlorophyll absorption band in the red region.

It does appear that the fluorescence is probably due more to chlorophyll than to phycoerythrin, or that the fluorescence spectrum of phycoerythrin in intact algae is very different indeed from the fluorescence spectrum of

the extracted material. We are indebted to Professor Robert Livingston for the suggestion that phycoerythrin and chlorophyll may in the intact algae be in combination with each other thus influencing the fluorescence spectrum of the complex and facilitating the transfer of energy from one to the other. Professor L. R. Blinks has suggested that the 655 mμ maximum is due to the presence of phycocyanin in the algae; an explanation which we have accepted as the most likely after seeing some of his unpublished absorption curves of similar algae. The maximum at 710 mμ would then presumably be due to chlorophyll fluorescence. Its position is, however, quite different from the 675 mμ found in chlorella fluorescence (**12**).

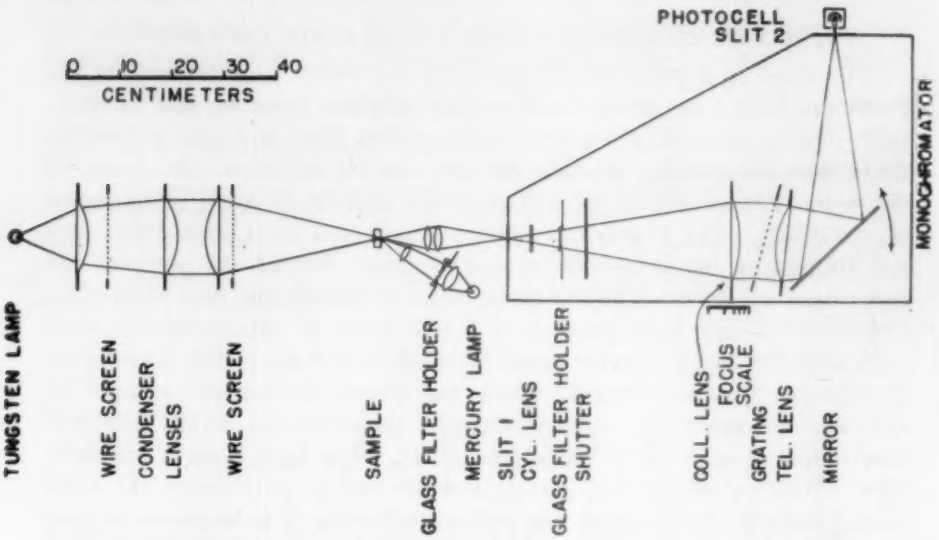

FIG. 5. The apparatus used to measure the fluorescence spectra of the intact algae by the comparison of its emission with that of a standardized tungsten lamp.

The fluorescence spectrum of the extracted material, measured with a slight modification of this apparatus, is presented in figure 2 in comparison with its absorption spectrum. In measuring the fluorescence spectra of the extracts a greater intensity was obtained by illuminating one corner of the vessel containing the extract and taking the fluorescent light out at right angles from the same corner. The cell containing the extract was placed adjacent to the entrance slit of the monochromator and illuminated from the side by the mercury lamp. Radiation from the standard lamp was arranged to pass through the same optical system as the fluorescent light, as in the original apparatus. We have determined the fluorescence curves for both the water extract containing phycoerythrin and the methyl alcohol extract containing chlorophyll. By shifting the chlorophyll fluorescence spectrum so that its maximum comes at 707 mμ and adding this curve to the fluorescence spectrum for phycoerythrin we are completely unable to

reproduce the shape of the fluorescence curves of the intact cells. It must therefore be concluded that the fluorescence spectrum of the phycoerythrin and of the chlorophyll is widely different in the intact algae from its shape in extracts, or that other pigments not present in either the water or the methyl alcohol extracts are causing some of the fluorescence of the intact material. The determination of the fluorescence curves of algae when excited by various wavelengths has not yet been attempted since it would require two monochromators and a more sensitive photocell. Until this can be done it will probably be difficult to come to a definite conclusion as to whether or not the energy absorbed by phycoerythrin is used directly for photosynthesis or is first transferred to chlorophyll.

The behavior of water extracts of algae when illuminated in the presence of dyes

The dye phenol-indophenol has been shown by Holt and French (**6, 7**) to be reduced to the leuco form when illuminated in the presence of active chloroplasts of spinach with a concomitant evolution of oxygen. This is believed to take place by means of a part of the photosynthesis system of the cell. Chloroplasts from our first shipment of *Gigartina radula* were not active in the reduction of the dye. A very slight activity was obtained from the Ulva chloroplasts. The extract of phycoerythrin from the first batch of Gigartina appeared to lose color when illuminated with added dye at pH 6.5. At this pH phenol-indophenol and phycoerythrin are both pink. Further investigation using slightly alkaline solutions of the dye which are blue or acid solutions of 2,6-dichlorophenol-indophenol showed that it was not the dye but the phycoerythrin which was bleached upon illumination in such solutions. Dye reduction experiments were later made with fresh phycoerythrin extracts of algae which had been shown to be photosynthetically active, again with negative results.

Summary

The absorption and fluorescence spectra of several marine algae are presented. The fluorescence yield in *Gigartina radula* and in *Iridaea sp.* is greater from green light than from red light. The fluorescence spectra of two red algae show a band at 575 mμ corresponding to phycoerythrin fluorescence, and also peaks at 675 mμ and 710 mμ.

It is a pleasure to thank the Graduate School of the University of Minnesota for grants which made this work possible. Drs. C. O. Rosendahl and John H. Moore gave invaluable assistance in the identification of the algae used. The photosynthesis experiments and the dye reduction experiments were done by Dr. A. S. Holt to whom we are also greatly indebted.

Department of Botany
University of Minnesota
Minneapolis, Minnesota.

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PHYSIOLOGICAL STUDIES OF WEED SEED GERMINATION

ROBERT O. BIBBEY

(WITH THREE FIGURES)

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The persistence of many annual and winter annual weeds on the grain farms of Western Canada is closely related to the tendency of their seeds to remain viable in the soil under conditions favorable for the germination of most crop species. Irrespective of competing ability, prolific seeding, and other plant habits, it is only by the relatively narrow germinative responses of its seeds that an annual species can persist under frequent summer-fallowing, spring and fall cultivation, and the natural hazards of low winter temperatures, drought, and plant competition.

DARLINGTON (**9**) and TOOLE and BROWN (**23**) have reported two of the classical experiments on the longevity of weed seeds. They showed that seeds of many species may live in a moist soil for several decades without germinating. TOOLE and BROWN point out that while the seeds of many Leguminoseae had to be scarified at the completion of the test before they would germinate, seeds of a number of species that had remained in the soil for 39 years had seed coats which were pervious to and had taken in water during the test.

A viable seed which is not in active growth is considered as being in a dormant condition. A seed may be in a state of dormancy because of the lack of one or more essential environmental conditions for its development, a condition termed in this paper "environmental" dormancy. Or the seed may be under a satisfactory germinative environment but be in a state of dormancy because of some physiological condition. This is considered as "inherent" dormancy. The terms "environmental" and "inherent" as applied to dormancy are synonymous with the terms "induced" and "natural" as used by BRENCHLEY and WARINGTON (**3**). They are preferable, however, since they are more descriptive of the condition. Also, the term "induced" was used previously by KIDD and WEST (**16**) in discussing the type of dormancy which developed in seeds of *Brassica alba* in high partial pressures of carbon dioxide. The two types of dormancy, inherent and environmental, are not always as distinct as they would at first appear, since the environmental requirements for the germination of a particular seed may vary somewhat with its degree of inherent dormancy.

CROCKER (**8**) divides inherent dormancy into "primary" and "secondary" types. Primary dormancy usually exists from maturity of the seed until it is ready to germinate for the first time. This type of dormancy is reported as being due to a seed coat which interferes with water adsorption, gaseous exchange, or expansion by the embryo, or to a direct

physiological condition of the embryo. A readily germinable seed which is under an adverse germinative environment may revert into secondary dormancy. KIDD (15) and KIDD and WEST (16) have shown that seeds of *Brassica alba* were thrown into secondary dormancy in the presence of high partial pressures of carbon dioxide. THORNTON (22) emphasizes reduced partial pressures of oxygen as a major factor in the establishment of secondary dormancy. DAVIS (10), working with Xanthium, found that restricted respiration at high temperatures readily induced secondary dormancy in seeds of that weed, and considers that the development of embryo dormancy during the summer and after-ripening during the winter is probably a general phenomenon with seeds of wild plants in the Temperate Zone. Environmental factors which may influence the germination of weed seeds in the field are temperature, aeration, moisture, and for some seeds, light. As well as playing a major role in environmental dormancy, these factors may also influence the after-ripening of seeds which are in a dormant condition.

Temperatures are too low for five months of the year in Western Canada to permit appreciable germination of weed seeds. However, low temperatures and alternate freezing and thawing have been shown by many workers to hasten the after-ripening of dormant seeds. CHEPIL (4, 5) found that this effect was pronounced in Saskatchewan, many species having their peak emergence in the spring. MARTIN (19) reports that temperatures around 0° C. were most effective in softening hard seeds of *Melilotus alba*. While weeds like *Amaranthus retroflexus* have been shown (2) to germinate when the soil is warm, many other weeds germinate under low temperature conditions in early spring.

Increase in germination of weed seeds when a compact soil is opened by tillage is generally attributed to better aeration. Oxygen and carbon dioxide were shown by CROCKER (7), THORNTON (22), KIDD (15), and KIDD and WEST (16) to be closely associated with primary and secondary dormancy, while seeds of different species were shown by HUTCHINS (14) to have different oxygen requirements for germination. The general picture as it pertains to weed seeds in agricultural soils is, however, somewhat obscure.

A number of workers have shown that the wetting and drying of weed seeds has a marked effect on overcoming seed dormancy. LEWIS (17) considers that drying of weed seeds on the soil surface is a major factor in after-ripening, and suggests that prolonged dormancy will occur if seeds are covered by tillage before this drying process takes place. DONEEN and MACGILLIVRARY (11) found that seeds would germinate at moistures as low as the wilting point of the soil. Unpublished data by the author, however, show that development at such moistures is very slow and probably of no significance in the field. Lack of moisture is a major factor limiting germination in the soils of Western Canada, since the moisture of the surface soil is below the wilting percentage during much of the growing season.

Light has been shown by GARDNER (12), HUTCHINGS (13) and others to be essential for the germination of seeds of a number of species. Tests showed that the seeds used in the experiments reported in this paper did not require light for optimum germination.

It was found in an earlier study (2) that relatively little was known about the field germination of most of the common weeds. While their seeds responded to various cultural treatments in different ways, the reasons for these responses were obscure. The study, therefore, was continued, and the physiology of weed seed germination in relation to the different environmental factors which may be met with in the soils of Western Canada was closely followed.

Materials and methods

The field studies were made on the Investigational Field of the Field Husbandry Department, University of Saskatchewan, Saskatoon, Canada.

TABLE I

AVERAGE MONTHLY MAXIMUM AND MINIMUM TEMPERATURES AND PRECIPITATION AT SASKATOON, SASKATCHEWAN, AUGUST 1935 TO DECEMBER 1941*

	JAN.	FEB.	MAR.	APR.	MAY	JUNE
Max. temp.	34.8	38.1	48.3	74.6	88.4	90.3
Min. temp.	−34.0	−37.5	−11.9	4.6	25.5	35.9
Prec. (in.)†	1.02	0.97	0.47	0.45	1.30	2.55
	JULY	AUG.	SEPT.	OCT.	NOV.	DEC.
Max. temp.	97.5	94.2	84.4	72.4	49.3	44.4
Min. temp.	44.1	37.2	25.8	10.1	−13.9	−30.4
Prec. (in.)†	1.48	1.21	0.96	1.19	0.94	0.86

* Courtesy of the Dept. of Physics, University of Saskatchewan.
† Total precipitation—11.29 inches.

The climate of this area is characterized by extremes in temperatures during the winter and summer, and by relatively low precipitation. The data in table I show the average monthly maximum and minimum temperatures, and precipitation for the period 1936–1941, during which time the field studies were made. Most of the precipitation from November to March is in the form of snow, and much of it is lost by drifting and evaporation. A heavy snow cover in the fall, however, acts as an insulation blanket and protects the soil from extreme temperatures and excessive drying during the winter. The melting of a heavy snow cover in March provides favorable moisture for the spring germination of weed and crop seeds. Usually, the soil thaws the first or second week in April and the seeding of spring grains is in full operation by the end of the month. June and July, on the average, are the months of highest precipitation with occasional temperatures over 100° F. The crop at this time is making its maximum growth. Harvesting generally starts early in August. Since the crop normally leaves the soil dry, favorable fall precipitation is of value as

reserve moisture for the following year and as a stimulant for the fall germination of many weed seeds. Usually the ground surface is frozen nightly during October and permanently early in November. The soils used in these field tests are classified as Asquith fine sandy loam, Elstow silty clay loam, and Elstow clay.

The initial studies were made on weed seeds which had been in the soil for a number of years. To obtain samples of these seeds about ten pounds of soil, composite of at least six locations on the plot, were obtained. This sample was washed with a stream of water through a fine zinc screen, care being taken to avoid damage to the weed seeds in the washing process. Small seeds were placed to germinate on moist blotting paper in Petri dishes, while larger seeds like those of *Avena fatua* were germinated half an inch deep in moist sand. All seeds were germinated at laboratory temperatures which ranged from 20 to 26° C. The seeds on blotting paper were considered to have germinated when their radicles broke through the seed coats. Emergence of the plumule above the sand was considered as germination in the case of the Avena seeds. SNEDECOR (**21**) was used as a guide in the statistical procedure.

In this work seeds of *Thlaspi arvense* (L.) (stinkweed), *Brassica arvensis* (L.) Ktze. (wild mustard) and *Avena fatua* (L.) (wild oats) were selected for particular study since they are three of the most troublesome annual or winter annual weeds on the grain growing farms of Western Canada.

LABORATORY STUDIES

The effect of oxygen and carbon dioxide on the germination of weed and crop seeds was studied, using a mercury sealed respiration flask developed by LOOMIS (**18**). The lower portion of the neck of a 1,000 ml., wide-mouth Erlenmeyer flask is restricted so that a No. 8 two-hole rubber stopper will fit securely into the recessed opening. Outlets to the flask consist of two pyrex capillary tubes with mercury seals. When the sample is in place, the flask is stoppered and mercury is poured into the neck of the flask to cover the rubber stopper. The advantage of the flask is that all outlets are sealed with mercury against the loss of carbon dioxide, and yet by lowering the mercury in the outlet seal samples of air may be readily taken.

Air containing a low oxygen pressure was obtained by adsorbing the oxygen from normal air in a closed container with alkaline pyrogallol solution. Carbon dioxide was produced in a Kipp generator with dilute hydrochloric acid and marble chips. After washing in distilled water the carbon dioxide was collected by water displacement in a 2,500 ml. bottle. For a desired oxygen concentration the respiration flask was evacuated to a predetermined tension and refilled with low oxygen air from the stock supply. For the lower oxygen pressures the evacuation procedure had to be repeated several times. With practice, a close approximation to the desired oxygen concentration could be readily obtained.

Carbon dioxide was drawn from the stock bottle into a gas burette and the desired amount forced into the respiration flask. If a carbon dioxide-free air was desired, a tube of sodium hydroxide solution was placed in the flask. Analysis of the air in the respiration flask gave a final check on the gas percentages. Some difficulty was experienced with the carbon dioxide on this first analysis, since it was not quickly miscible with the other gases in the flask. By drawing and replacing the sample several times when making analysis, this difficulty was overcome.

The seeds were placed to germinate at different gaseous pressures on moist blotting paper at the bottom of the respiration flasks. Unless otherwise noted, the tests were made at laboratory temperatures in diffused light. The length of test varied with the response of the seed. Upon completion, a final analysis was made on the atmosphere in the respiration flask, final notes taken, and any ungerminated seeds placed to germinate in Petri dishes at normal gas pressures. The seeds used in this series of tests were grown during 1945 at Saskatoon, Canada. The crop seeds were harvested and threshed in the normal manner. The weed seeds were threshed with a small-plot threshing machine and were stored over winter in an unheated shed. The seeds were chosen for their high germinability and their readiness to grow. Seeds of the following crops and weeds were used: Apex wheat (spring) (*Triticum vulgare* Vill.), wild oats (*Avena fatua* L.), Indian mustard (*Brassica juncea* (L.) Cosson) wild mustard (*Brassica arvensis* (L.) Ktze.), stinkweed, (*Thlaspi arvense* L.).

Results

The dormancy of weed seeds in the soil

The success of weed control measures in eliminating weed seeds from the soil depends, to a large extent, on the germination of the seeds. Although this fact is widely recognized by agriculturists, and many studies of after-ripening and germination of weed seeds have been made in the laboratory, little is known about the dormancy of weed seeds in the soil. An attempt was made, therefore, to determine the types of dormancy affecting weed seeds that had lain in the soil for a number of years.

The results of germination tests made on seeds of *Thlaspi arvense* and *Brassica arvensis* recovered after two years in a silty clay loam soil are presented in table II. The seeds were mixed with the top three inches of the soil in September, 1935, and the area remained undisturbed, except for tillage half an inch deep to control seedlings, from that date until the samples were taken.

The exceptionally high viability of the Thlaspi seeds after lying in the soil over thirty months, and the spontaneity of their germination are shown by the data. Since nearly 100 per cent. of the dormant seeds of this weed were readily germinable under favorable germinative conditions, they were apparently in a condition of environmental dormancy in the soil.

TABLE II

THE GERMINATION OF SEEDS OF *Thlaspi arvense* AND *Brassica arvensis* UPON REMOVAL FROM THE SOIL AFTER PERIODS OF DORMANCY OF 31, 36, 43 AND 44 MONTHS*

DATE WEEDS WASHED FROM SOIL	DEPTH OF SAMPLING INS.	THLASPI ARVENSE		BRASSICA ARVENSIS	
		NO. OF SEEDS	% GERM. AT 5 DAYS	NO. OF SEEDS	% GERM. AT 5 DAYS
27 April 1938 (31 months)	0–1	64	100	28	72
	1–3	299	100	49	86
	1–3	250	100	86	91
	1–3	269	100	95	84
9 Sept. 1938 (36 months)	0–3	211	98	35	40
	0–3	203	92	47	30
20 April 1939 (43 months)	0–3	67	98	25	92
	0–3	80	100	28	82
	0–3	49	96	.....	.....
20 May 1939 (44 months)	0–3	24	100	7	86
	0–3	33	97	33	97
	0–3	20	100	20	90

* Seeds mixed with soil in the field September 6, 1935.

Seeds of *Brassica arvensis* germinated well in the spring but showed apparent secondary dormancy when tested in September. Although the latter samples were kept under germinative conditions for over seven months, little further germination occurred. By the following spring the Brassica seeds in the soil were again highly germinable, and were apparently in environmental dormancy at this time.

Many seeds of *Melilotus alba* as well as Brassica and Thlaspi were recovered from the soil in the above experiment. Records show that a seed crop of sweet clover was grown on the soil in 1927 and 1928. Since then

TABLE III

THE GERMINATION OF SEEDS OF *Melilotus alba* UPON REMOVAL FROM THE SOIL AFTER PERIODS OF DORMANCY OF 9½, 10, AND 10½ YEARS*

DATE SEEDS WASHED FROM SOIL	DEPTH OF SAMPLING, INS.	NO. OF SEEDS	NO. GERMINATED AFTER 5 DAYS
27 April 1938	0–1	161	1
	1–3	300	3
	1–3 (scarified)	25	25 (100%)†
9 Sept. 1938	0–3	100	7
20 April 1939	0–3	197	11
	0–3 (scarified)	31	31 (100%)
20 May 1939	0–3	382	13

* Seeds shattered on soil in Aug. 1928.

† These seeds remained under germination conditions with hard seed coats from 27 April until 7 August 1938. They were then scarified with a pin, and germinated 100 per cent. within three days. The remainder of the seeds were carried until 28 April 1939 without further germination.

the field had been under different cultural and cropping treatments, all of which omitted sweet clover. The distribution and quantity of seed present precluded the possibility of it being transported on to the area. The seeds were placed to germinate on moist blotting paper in Petri dishes at laboratory temperatures. The results from these tests, presented in table III, show that most of the Melilotus seeds recovered were in a "hard" condition. Since they absorbed water readily and germinated upon scarification, it is fairly safe to assume that they were still in a condition of primary dormancy because the seed coat prevented access of water to the embryo. One hundred per cent. germination was obtained when the seeds

TABLE IV

GERMINATION AT 5 DAYS OF SEEDS OF *Brassica arvensis* WHEN REMOVED FROM DIFFERENT DEPTHS IN THE SOIL AT INTERVALS DURING THE GROWING SEASON.*

DEPTH IN INCHES	DATES SAMPLES TAKEN 1938								MEAN
	APRIL 8		MAY 11		JUNE 14		SEPT. 8		
	a	b	a	b	a	b	a	b	
0–1 No. of seeds	14	15	10	2	6	5	18	9	9.9
% germ.	42.8	40.0	20.0	100.0	0	40.0	100.0	77.8	54.5
1–2 No. of seeds	34	51	41	25	8	13	15	2	23.6
% germ.	61.8	58.8	56.2	56.0	62.5	46.1	60.0	100.0	58.2
2–3 No. of seeds	100	66	96	66	61	65	44	33	66.4
% germ.	87.0	66.6	88.5	84.8	87.0	74.0	84.1	88.0	82.7
3–4 No. of seeds	101	117	110	83	114	99	95	72	98.9
% germ.	82.2	72.6	84.5	92.8	83.4	88.0	71.6	87.5	82.3
4–5 No. of seeds	116	131	137	132	148	111	130	135	130.0
% germ.	83.6	79.3	88.2	86.3	95.1	90.0	90.0	94.0	88.5
Mean % germination at 5 days	75.6		83.6		85.1		86.2		

* Seeds placed in soil September, 1936.

were scarified. The freezing and thawing and low temperatures of a Western Canadian winter "softened" less than 4 per cent. of the seeds which had withstood over ten years of weathering in the soil. Samples of soil taken seven years later, in December 1945, still contained sweet clover seeds which germinated freely upon scarification.

In a second study, in co-operation with the Research Laboratory of Plant Ecology of the University of Saskatchewan, seeds of *Brassica arvensis* were mixed with a silty clay loam and placed in wooden soil boxes with galvanized wire bottoms in September, 1936. The boxes were dug into the surface soil, the wire screen bottoms allowing direct moisture contact with the sub-soil. Results of germination tests on the Brassica seeds after lying at different depths in the soil from nineteen to twenty-three months are presented in table IV.

The data show that the seeds of Brassica which had lain 2 to 5 inches

deep for over eighteen months in the soil were highly germinable throughout the season. Most of the germinable seeds from the 0 to 2 inch depths had germinated, and only a few of possibly the most dormant seeds survived. Although the seeds at the lower depths were highly germinable, the survival of the seeds was greater at these depths. Germinability of the seeds increased as the season progressed and no definite evidence of secondary dormancy was shown.

Additional evidence of the dormancy of weed seeds in the soil was obtained from a test where seeds of *Thlaspi arvense* and *Brassica arvensis* were mixed with the top four inches of a fine sandy loam and a clay soil in August, 1940. When recovered from the soil five years later, in November, 1945, Thlaspi seeds from the 1 to 3 inches depth germinated 90.0 ± 3.3 and 87.0 ± 3.2 per cent., and the Brassica seeds 79.4 ± 3.6 and 98.0 ± 5.0 per cent. from the two soils, respectively.

The data presented above indicate that seeds of these weeds may lie viable in the soil for several years. During a large part of this time they may be readily germinable and apparently in a condition of environmental dormancy. Seeds of *Melilotus alba*, on the other hand, appear to persist in the soil by virtue of their hard seed coats, and were still in a condition of primary dormancy after seventeen years in the soil. While there was some evidence of secondary dormancy in the Brassica seed, none was found in that of Thlaspi. The data suggest that secondary dormancy was not a major factor in the longevity of the seeds in the soil under the conditions of these tests.

After-ripening of weed seeds

To outline effective weed control measures it is not only important to know the type of dormancy responsible for the longevity of the weed seeds in the soil, but also how soon that type of dormancy is established after the seed shatters from the plant. The after-ripening of seeds of *Thlaspi arvense*, *Brassica arvensis*, and *Avena fatua* was, therefore, studied in the following environments:

1. 0–1 inch deep in a fine sandy loam soil.
2. 1–3 inches deep in a fine sandy loam soil.
3. 0–1 inch deep in a clay soil.
4. 1–3 inches deep in a clay soil.
5. On the ground surface of a silty clay loam soil.

Figure 1 shows the results of germination tests made on seeds of *Thlaspi arvense* recovered from these different environments during the fall of 1940 and spring of 1941. While marked and significant differences in after-ripening were found under the different conditions, the data are for one year only and the fall of 1940 was somewhat dry. One generalization, however, can be made. Even though the samples of Thlaspi seed varied widely in fall after-ripening, marked changes took place during the winter so that

by spring most of the seeds had lost their primary dormancy and were readily germinable.

Further evidence of this winter after-ripening is found for seeds of *Avena fatua* in figure 2. Under the conditions of the fall of 1940, the seeds on the ground surface after-ripened much faster than those in the two soils. However, by spring the majority of the seeds under test were readily germinable. Figure 2 shows also the difference which may be found in

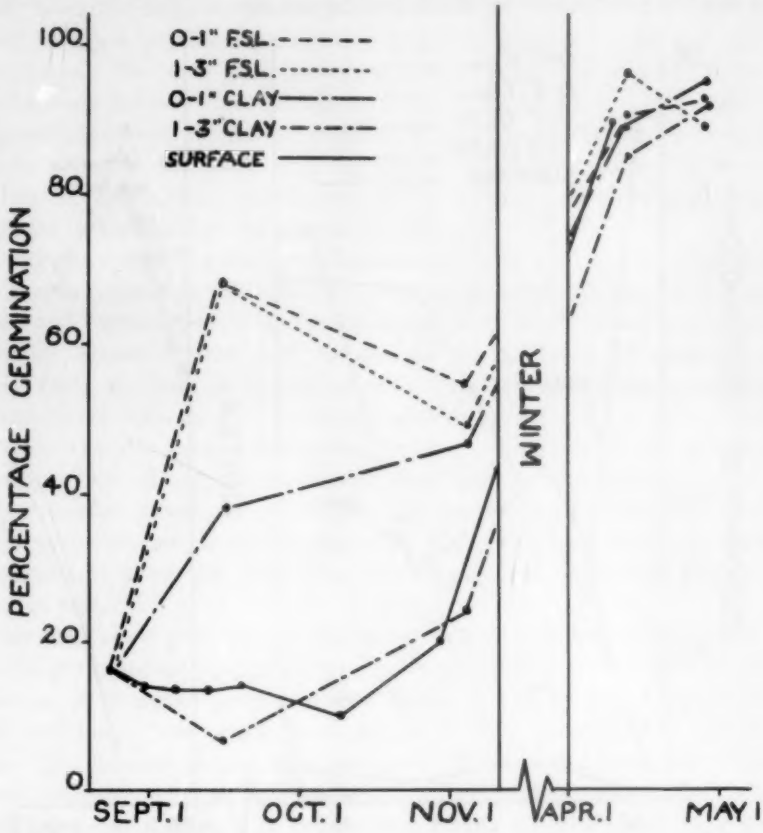

FIG. 1. Percentage germination at 5 days of seeds of *Thlaspi arvense* after-ripened 0–1 or 1–3 in. deep in fine sandy loam or clay soil, or on the ground surface. (Standard Errors of Means for soil after-ripened samples: Sept. 14 = 3.5%, Nov. 3 = 7.2%, April 12 = 2.3%, April 27 = 1.7%. SE_m for all dates of surface after-ripened seeds = 12.5%.)

Avena seeds at harvest maturity. The seeds used in the surface study in 1940 and 1941 were collected from the same area of a farmer's field. The 1940 seed germinated 5 per cent. when harvested while the 1941 seed germinated 95 per cent. at the same stage. Both samples of seed appeared equally mature.

The response from seeds of *Brassica arvensis* was similar to that of Thlaspi and Avena except that while much after-ripening took place dur-

ing the winter, marked after-ripening also occurred between April 12 and April 27 the following spring. The above results indicate that the conditions of late fall, winter, and early spring in Saskatchewan are major factors in the after-ripening of seeds of *Thlaspi arvense, Brassica arvensis,* and *Avena fatua.* The effect of variable environmental conditions during the fall are masked by the winter (and early spring) after-ripening, and most of the seeds which were in a primary dormant condition in the fall were readily germinable by the following spring.

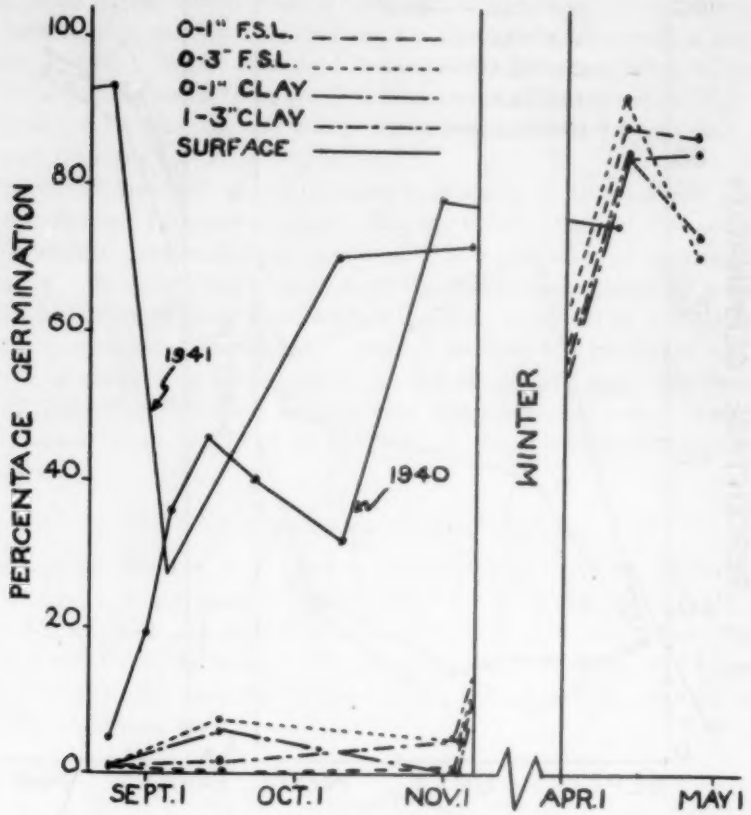

FIG. 2. Germination at 15 days of seeds of *Avena fatua* after-ripened 0–1 or 1–3 in. deep in a fine sandy loam or clay soil, or on the ground surface. (Average of 4 replicates, insufficient germination in fall for statistical analysis. SE_m for sampling April 12 = 5.5%, April 27 = 3.2%. SE_m for all dates of surface after-ripened 1940 seed = 8.1%.)

THE EFFECT OF OXYGEN AND CARBON DIOXIDE ON THE GERMINATION OF WEED AND CROP SEEDS

When a compact soil is loosened by tillage, or grass land plowed, a heavy crop of weed seedlings often develops. The explanation usually given for this development is that cultivation increases the soil aeration and stimulates germination. Earlier in this study evidence was presented which

showed that seeds of *Thlaspi arvense* and *Brassica arvensis*, which were not in a condition of inherent dormancy, would remain for years buried in the soil without germinating. Poor aeration would appear to be a possible factor influencing the germinative behaviour of these seeds. If poor aeration is the cause of these seeds lying dormant in the soil, the question arises as to why only particular species display these dormant tendencies while many closely related species of both weeds and crops will germinate readily from the same environment.

Soil aeration implies the exchange of gases between the air and the soil atmospheres. For seed germination, equilibrium of oxygen and carbon dioxide between the two atmospheres is of major importance. Although these two gases have been extensively studied in relation to the after-ripening of seeds in primary dormancy and the induction of secondary dormancy in non-dormant seeds, their relationship to the germination of readily germinable seeds in the soil has received scant attention.

Using the mercury sealed respiration flask described above, experiments were run to determine the effect of different atmospheric concentrations of oxygen and carbon dioxide on the germination of seeds of different species. Particular attention was paid to species the seeds of which germinate readily from the soil, as compared with those which display pronounced environmental dormancy. The seeds chosen for study had after-ripened, and were readily germinable under normal oxygen and carbon dioxide pressures. A summary of the results from germination tests made at (1) varying partial pressures of oxygen and no carbon dioxide, (2) normal oxygen and varying partial pressures of carbon dioxide, and (3) varying concentrations of oxygen with from 4.8–7.2 per cent. carbon dioxide is presented in table V.

The data show that the species were divided into two groups with respect to germination under adverse gaseous conditions. The first group, consisting of *Brassica juncea, Avena fatua,* and *Triticum vulgare,* germinated well under low pressures of oxygen or high pressures of carbon dioxide. The second group, consisting of *Brassica arvensis* and *Thlaspi arvense,* germinated poorly when subjected to gaseous pressures which deviated far from normal. It is significant that seeds of both *B. arvensis* and *T. arvense* have been shown to display pronounced environmental dormancy in the field, while those of *B. juncea* and *T. vulgare* germinate readily, and thus are relatively short lived in the soil. Seeds of *Avena fatua* may remain viable for several years in the soil. It is possible, however, that the response to the gases of the Avena seeds used in these tests was affected by the stage of their after-ripening, an effect which has previously been shown by Atwood (1).

The responses of the two closely related species *B. arvensis* and *B. juncea* to the gases is shown graphically in figure 3. All regression lines except that for *B. juncea* and oxygen are statistically significant. The broken line of figure 3 represents an extension of the variable oxygen—(4.8 to 7.2 per

cent.) carbon dioxide curve. A sudden break in germination occurred at about 5 per cent. oxygen, and while this was not calculated in the regression, it was possibly a typical response of the material. The curves for *B. arvensis* show marked inhibition with less than 16 per cent. oxygen or more than 5 per cent. carbon dioxide, and almost total inhibition with less than 12 per cent. oxygen or over 10 per cent. carbon dioxide. A combination of

TABLE V

SUMMARY OF THE PERCENTAGE GERMINATION OF WEED AND CROP SEEDS IN DIFFERENT ATMOSPHERIC CONCENTRATIONS OF OXYGEN AND CARBON DIOXIDE

% O_2	% CO_2	THLASPI ARVENSE	BRASSICA ARVENSIS	BRASSICA JUNCEA	AVENA FATUA	TRITICUM VULGARE
Variable oxygen						
20.4–20.4	0	88	65	100	98	100
16.1–18.0	0	72	66	.....	.....	.....
14.1–16.0	0	51	26	100	100	100
12.1–14.0	0	52	32	.....	100	.....
10.1–12.0	0	15	3	.....	.....	.....
8.1–10.0	0	6	8	96	96	.....
6.1– 8.0	0	0	0	98	92	.....
Regression line E† =		1.864X – 15.0**	1.454X – 13.0**	0.0896X + 23.4	.....	.....
s_b ‡ = ±		0.20	0.19	0.039	.....	.....
Variable carbon dioxide						
20.4–20.4	0.0– 0.0	86	70	100	94	92
19.1–19.1	4.0– 4.9	64	64	100	.....	96
19.4–19.0	5.0– 5.9	70	48	98	92	96
19.3–19.3	6.0– 7.0	52	40	.....	96	.....
19.0–19.0	8.0– 8.9	8	40	92	92	.....
18.8–18.8	9.0– 9.9	20	40	.....	.....	92
18.3–18.3	10.0–13.9	.....	.....	84	92	88
17.8–16.9	14.0–15.9	2	20	94	88	88
17.1–15.9	16.0–18.0	0	16	.....	92	.....
15.5–15.0	24.0–26.0	.....	.....	.....	90	.....
Regression line E† =		21.9 – 1.529X**	16.6 – 0.8300X**	25.3 – 0.2147X*	.....	.....
s_b ‡ = ±		0.23	0.08	0.08	.....	.....
Variable oxygen, 4.8–7.2 per cent. CO_2						
20.4	0	88	65	100	98	100
18.1–20.0	4.8–7.2	64	45	98	94	96
16.1–18.0	"	42	40	.....	.....	.....
14.1–16.0	"	20	24	98	98	.....
12.1–14.0	"	17	8	.....	.....	96
10.1–12.0	"	12	8	98	96	.....
8.1–10.0	"	.....	.....	.....	68	.....
6.1– 8.0	"	.....	.....	80	68	96
4.8–	"	.....	.....	4	.....	.....
3.6–	"	.....	.....	.....	.....	96
Regression line E† =		1.690X – 17.56**	1.386X – 14.9**	0.2791X + 19.8*	.....	.....
s_b ‡ = ±		0.31	0.26	0.10	.....	.....

† Regression of germination on gas concentration was calculated from the original data.

** Odds 100: 1, * odds 20: 1 that regression coefficient is significant.

‡ s_b = Standard error of regression b.

reduced oxygen pressures and 4.8 to 7.2 per cent. carbon dioxide appeared to make the effect of the adverse gaseous conditions more pronounced. *B. juncea* was relatively unaffected in the range of gas pressures used.

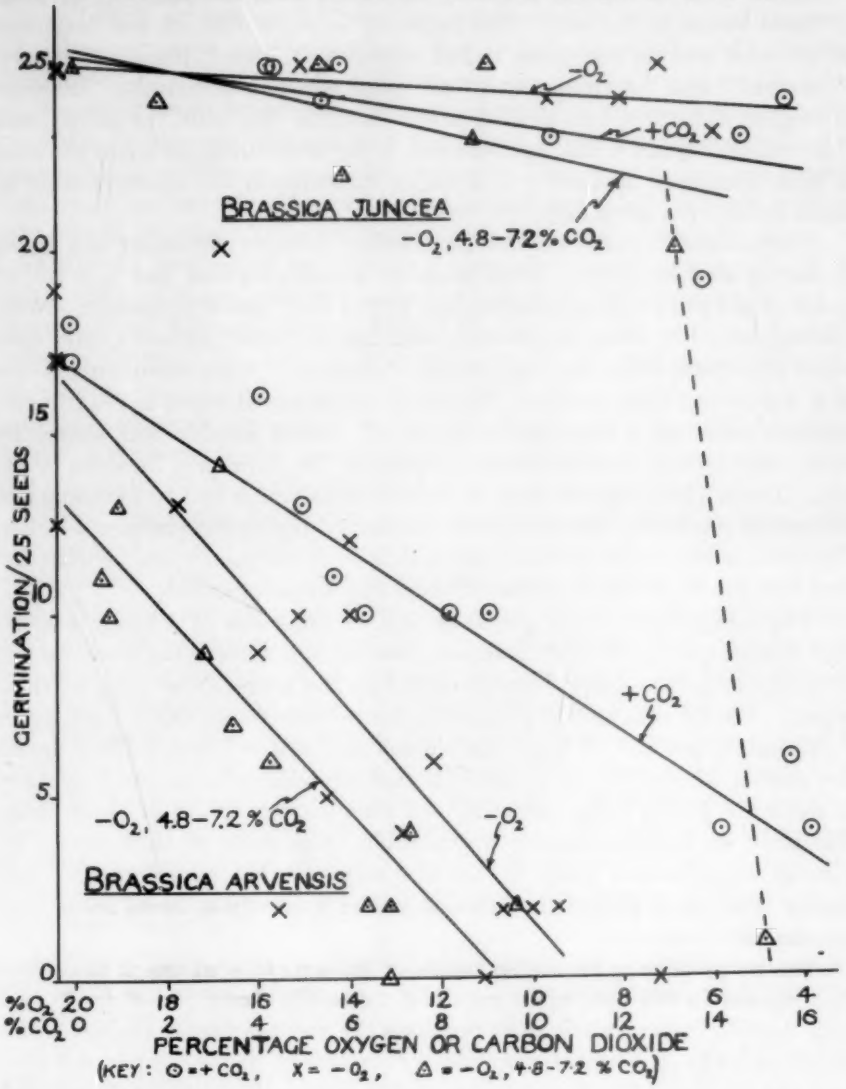

FIG. 3. Germination of seeds of *Brassica arvensis* and *Brassica juncea* under different partial pressures of oxygen and carbon dioxide.

Germination tests made on ungerminated seed recovered from the respiration flasks upon the completion of the above test were variable and inconclusive. There was, however, considerably more secondary dormancy in both the Brassica and Thlaspi seeds than was found in the field surveys.

Discussion

The persistence of many annual and winter annual weeds on the grain farms of Western Canada is closely associated with the longevity of seeds of these weeds in the soil. This longevity is dependent on the dormancy of the seed and its resistance to soil organisms. Two types of dormancy, "inherent" and "environmental" are emphasized in this study. Inherent dormancy is further sub-divided, after CROCKER (8), into "primary" and "secondary" types. The environment is the controlling factor in environmental dormancy, and often is a major influence in the after-ripening of seeds in inherent dormancy.

For seeds with coats impervious to water, primary dormancy is a major factor in seed longevity. Thus seeds of *Melilotus* which had lain for 17 years in the soil required scarification before they would germinate. With *Thlaspi arvense, Brassica arvensis, and Avena fatua,* however, our data show that most seeds showing primary dormancy after-ripen during the first winter and early spring. Secondary dormancy induced in readily germinable seeds by a high concentration of carbon dioxide was shown by KIDD (15) to be a possible factor influencing the dormancy of seeds in the soil. DAVIS (10) suggests that in nature seeds which fail to germinate in the spring are forced into secondary dormancy during hot summer weather. The data in this study indicate that this type of dormancy was of little importance in the longevity of the Thlaspi and Brassica seeds.

The exceptionally high germinability of the seeds of *Thlaspi arvense* and *Brassica arvensis* when washed from the soil after lying dormant for several years offers a new concept regarding the longevity of seeds of these weeds. Both weeds have a reputation for persistence on heavier soil types in Saskatchewan and in most other areas in the world where small grains are grown, which has led to the idea that pronounced inherent dormancy is the main factor influencing the soil germinating habits of their seeds. The more or less spontaneous germination from seeds of these weeds recovered after several years in the soil suggests that environmental dormancy is of much greater importance in the longevity of weed seeds than is generally recognized.

The persistence of readily germinable seeds of *B. arvensis* at the 2 to 5 inch depths in the soil, while germinating fairly readily from the 0 to 2 inch depths (table IV) is evidence that the environmental factors which maintain seeds in dormancy are more pronounced in the deeper soil than near the surface. The longevity of readily germinable seeds of *B. arvensis* and *T. arvense* in the fine sandy loam soil for over five years shows that these environmental factors are also operative in light soils.

Moisture and temperature were favorable for germination many times during the growing season, so that aeration suggests itself as the major factor influencing the germination of seeds in the soil. Support for emphasis on aeration was found in the close relationship between the germination of seeds of different species in the soil and their sensitivity to low oxy-

gen or high carbon dioxide concentrations in their germinative atmosphere. Species like *Brassica juncea* which germinated readily from the soil were found to be relatively unaffected by unfavorable gaseous environment, while species like *B. arvensis* and *T. arvense*, which display marked environmental dormancy, were very sensitive to low oxygen or high carbon dioxide pressures.

The main inhibition of seeds of *B. arvensis* and *T. arvense* occurred with less than 16 per cent. oxygen and more than 5 per cent. carbon dioxide. CLEMENTS (**6**), however, found few instances where the oxygen concentrations of the soil air were reported as less, or the carbon dioxide higher, than these concentrations. Only on flooded land, grass sod, or recently manured land, did the analysis of the soil air deviate very far from normal. HUTCHINS (**14**) probably obtained a better indication of soil aeration when measuring the diffusion of oxygen from the soil into porous cups placed at different depths. The amount of moisture in the soil and the depth of the porous cups were found to be the main factors affecting this diffusion. No data were obtained for carbon dioxide, but it is possible that the minimum oxygen requirements for germination determined for diverse seeds were also a measure of the carbon dioxide tolerance of these seeds.

When heavy rain falls on a soil, most of the pore space is filled with gravitational water. Soil air is largely excluded, except possibly for many small pockets trapped by the moisture and gases absorbed on the soil surface. The rain carries some dissolved oxygen into the soil, and this, coupled with the oxygen in the trapped air, is the main supply for seeds as well as micro-organisms. It is probable that this supply is quickly utilized. Any replenishment must come by aqueous diffusion from the soil surface, and HUTCHINS's data suggest that this is a relatively slow process, since it required two or three days for the oxygen supply in a soil to return to normal after the removal of a restriction to gaseous exchange at the soil surface. Since oxygen is not highly soluble in water, there is also the possibility that pronounced microbiological activity near the soil surface may "filter" the dissolved oxygen from the soil solution, and thus starve seeds and other organisms deeper in the soil.

Carbon dioxide is chiefly produced in a soil by the respiration of micro-organisms and plant tissues. Its concentration in the soil atmosphere is affected by its solubility in water, about 40 times that of oxygen. The soil solution will therefore act as a buffer, and the analysis of soil air will be somewhat misleading as to the quantity of carbon dioxide present. In a wet soil, carbon dioxide dispersion will be chiefly by aqueous diffusion, and, as with oxygen, micro-biological activity near the soil surface may influence this process by creating adverse diffusion gradients.

The point in a drying soil where direct gaseous diffusion may take place between the soil and outer atmospheres has not been determined. Soil moisture is probably in a continuous phase down to the field percentage, and it is not known whether a parallel, continuous phase of air exists in the

soil at the same time. Only when the soil air is continuous will many seeds with high oxygen requirements or low carbon dioxide tolerances have sufficient aeration for germination. The fact that these seeds may lie readily germinable in a soil for many years suggests that when aeration is sufficient, some other factor, (possibly moisture), has become limiting. If this is so, the continuous air phase may not be reached until the soil moisture is below the field percentage and approaching the wilting point.

Only one year's data are presented in the after-ripening study. It is, therefore, undesirable to draw too definite conclusions as to after-ripening in the different environments. There is, however, strong evidence from the data presented and from observations during other years, that irrespective of the after-ripening during the fall, marked after-ripening takes place during the winter and early spring. The majority of the seeds of *Thlaspi arvense, Brassica arvensis* and *Avena fatua* lose their primary dormancy during this after-ripening period and are readily germinable in the spring. It would appear to be a sound agricultural practice to have the seeds under a favorable environment for germination at this time.

Summary

1. Environmental influences on the after-ripening and dormancy of seeds of a number of species were studied in the field and laboratory.

2. "Environmental" dormancy is defined as the type of dormancy affecting a readily germinable seed when it is prevented from germinating by the absence of some essential factor or by the presence of some inhibitor; and "inherent" dormancy, embracing both primary and secondary types, as the dormancy existing in a seed which fails to germinate under apparently satisfactory germinating conditions.

3. Most primary dormancy disappeared from the seeds of *Thlaspi arvense, Brassica arvensis* and *Avena fatua* in the soil during the first winter and early spring after-ripening period. Although secondary dormancy was produced experimentally, none was found in any of the Thlaspi seeds in the field, and only isolated instances of it in the seeds of Brassica. After the first winter most seeds of these weeds were readily germinable in the soil, and apparently environmental dormancy was the major factor in their longevity.

4. Seeds of *Melilotus alba* were found to be still in a condition of primary dormancy after 17 years in the soil.

5. Seeds of *Thlaspi arvense* and *Brassica arvensis*, which display marked environmental dormancy, were shown to be much more sensitive to lower-than-normal oxygen or higher-than-normal carbon dioxide pressures than were seeds of *Triticum vulgare* and *Brassica juncea*, which are relatively short lived in a soil.

The writer wishes to acknowledge, with greatest appreciation, the valu-

able direction and assistance he has received throughout the course of this study from Dr. W. E. Loomis.

DIVISION OF BOTANY AND PLANT PATHOLOGY
SCIENCE SERVICE
DEPARTMENT OF AGRICULTURE
OTTAWA, CANADA

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RATE OF LEAF ELONGATION AS AFFECTED BY THE INTENSITY OF THE TOTAL SOIL MOISTURE STRESS[1]

C. H. WADLEIGH AND H. G. GAUCH[2]

(WITH SIX FIGURES)

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Recent studies (**1, 7, 9**) have indicated that an interrelationship exists between soil salinity and the soil moisture content in their effect on plant growth. These two factors may be logically evaluated in terms of the decrease in the specific free energy of the soil moisture. Thus, salinity may be expressed in terms of the osmotic pressure of the soil solution, and the soil moisture status with respect to surface force action is expressable in terms of the soil moisture tension in atmospheres. The sum of these two components has been termed the "total soil moisture stress" (**7**).

It is difficult to evaluate the moisture stress to which a plant is responding when grown on a given mass of saline soil. This moisture stress which is conditioning the entry of water into the roots will be largely affected by five variables: (a) the variation in salt distribution within the soil mass and its consequent effect on the variation in the osmotic pressure of the soil solution at a given moisture content; (b) variation in osmotic pressure in relation to change in moisture content; (c) variation in moisture tension in relation to moisture content; (d) variation in moisture content within the soil mass at a given time; and, (e) variation in total water content of the soil mass with time, i.e., over an irrigation interval. A method has been developed to integrate these variables as they obtain within a large container of saline soil (**8**). This method makes it possible to determine the relation of the theoretically uniform stress, S, throughout that part of the soil mass within which moisture movement into the plant is taking place, to the total moisture content, W, of the soil. That is,

$$S = f(W)$$

This stress is called the integrated soil moisture stress.

Since the moisture content of the soil supporting a growing plant changes continuously with time, the integrated moisture stress in the soil also changes with time. If the curve is obtained which shows the integrated stress as a function of time, then the average integrated stress over a given time interval will be that constant stress represented by a horizontal line on the chart having under it the same area as that subtending the curve.

[1] Contribution from the U. S. Regional Salinity and Rubidoux Laboratories, Riverside, California, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, in cooperation with the eleven Western States and the Territory of Hawaii.

[2] Principle Chemist, and formerly Associate Physiologist.

This average integrated stress is referred to as the average daily moisture stress, S_a. It may be formally expressed as

$$S_a = \frac{\int_{t_x}^{t_o} Sdt}{t_x - t_o}$$

where time, t, is expressed in days, and t_o and t_x are the limits of integration.

It has been observed with beans (7) and guayule (9) that there is a fairly close relationship between vegetative growth and the average moisture stress, if other factors affecting growth are not limiting and are kept as uniform as possible. Since the resultant growth response appears to be related to the average daily moisture stress, it should follow that daily variations in plant growth could be observed concomitant with the variations in moisture stress taking place over an irrigation cycle. It is the purpose of this report to present observations on this postulated relationship.

Methods

Cotton plants of the Acala P–18–C variety were grown individually in steel drums containing 100 pounds of adequately fertilized Fallbrook loam.

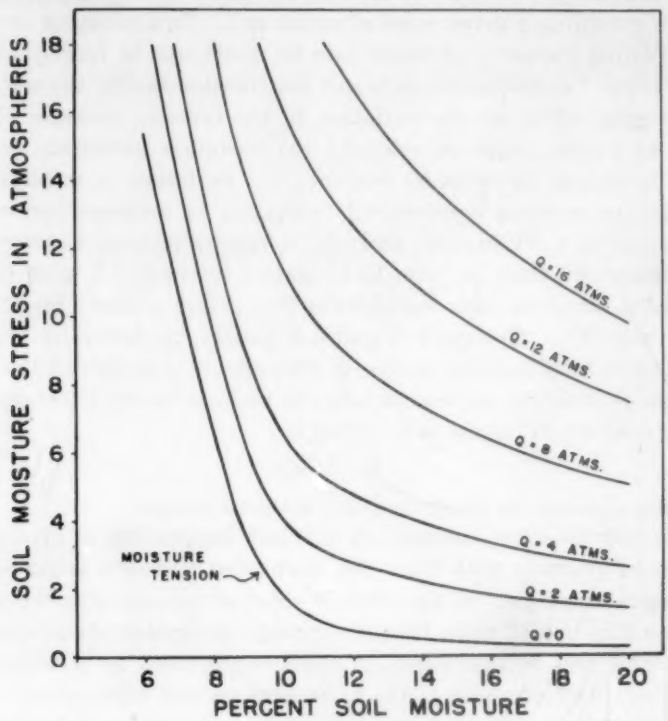

FIG. 1. The relation between moisture tension and moisture content together with variations in total moisture stress—tension plus osmotic pressure—when various amounts of salt, Q, have been added to the soil. In this case Q is taken as the osmotic pressure of the soil solution when the soil contains 12% moisture.

This soil has a field capacity of 18% moisture and the wilting percentage is 6.1. The relationship between moisture tension and moisture content is shown in figure 1, as well as the variations in total moisture stress with moisture content when various amounts of salt have been added to this soil. The "control" plants were grown on this nonsaline soil with no added salt other than fertilizer. The "saline" plants were grown on soil to which 0.2% NaCl had been added. The saline cultures were alternately surface and sub-irrigated in order to maintain a more nearly uniform distribution

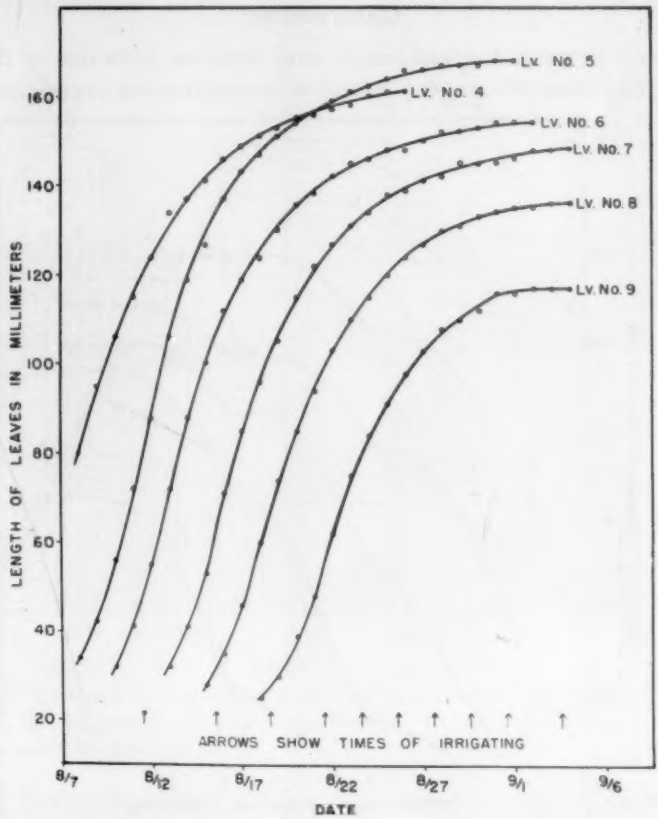

FIG. 2. Leaf length data obtained from one of the control plants.

of salt in the soil mass. After each irrigation, two cultures containing saline soils were discontinued and sampled with respect to salt distribution and moisture content. Soil salinity was evaluated in terms of the electrical conductivity of the extract from the saturated soil (**5**). The osmotic pressure of the soil solution at prevailing moisture contents was calculated by means of a previously ascertained relationship between these factors for this soil (**1, 5**). The drums of soil were weighed at 8 o'clock each morning. When the average moisture content of a given container of soil reached 12%, sufficient water was added to bring the average to 21%.

The length, L, of each leaf arising from the main axis of each plant was measured at the same time every morning during the course of this study. Thus, L was taken as a measure of plant size; and growth, G, may be defined for the present case as the rate of elongation of the leaves, or

$$G = dL/dt$$

This paper is concerned with the relation of growth to moisture stress, or

$$G = f(S).$$

Observations

Figure 2 presents the leaf-length data obtained from one of the control plants. The observations tend to follow smooth curves suggesting that the

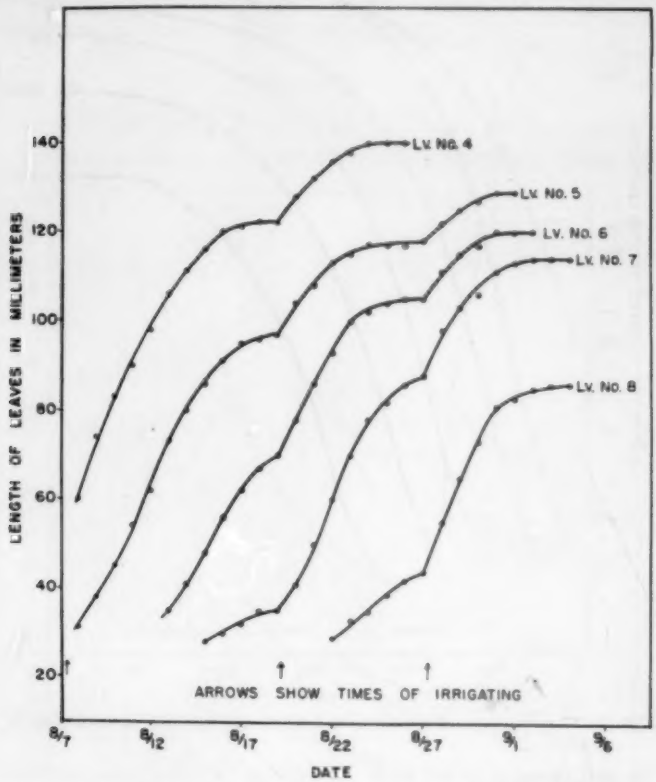

FIG. 3. Leaf length data obtained from one of the plants on saline soil.

minor variations present were not related to the irrigation schedule. That is, it appears that the small fluctuations in moisture stress, from 1 to 3 atmospheres, within the control cultures were without observable effect on rate of leaf elongation.

The typical observations on leaf elongation for one of the plants on saline soil are shown in figure 3. It should be noted that growth of an expanding

leaf just about ceased by the time the soil was ready for an irrigation. If the leaf was incompletely developed, elongation resumed at a fairly rapid rate immediately following an irrigation and nearly ceased by the time of the next irrigation.

The variation in the average moisture content with time is shown in figure 4. This is the same as the total water content in pounds, since the dry

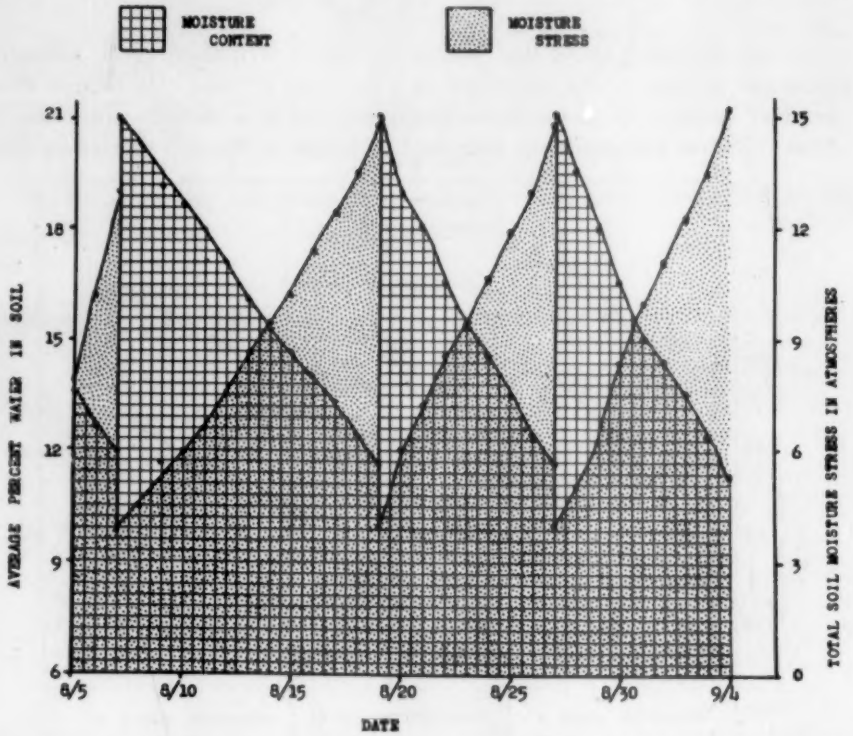

FIG. 4. Variation in average moisture content with time and the respective values derived for the integrated moisture stress for the culture of soil which produced the plant represented by figure 3.

weight of soil per container was 100 pounds. From the previously established relation of stress to water content it is possible to calculate the stress at each moisture content and thus represent the stress also as a function of time in this figure, i.e.,

$$S = f(t).$$

As pointed out elsewhere (9), the shape of these stress-time curves is specific for a given culture of soil during a given irrigation interval, in that they depend on the status of salt distribution within the soil and the rate of water removal from the soil.

The shapes of the leaf elongation curves within a given irrigation period are essentially parabolic. Hence, a second degree equation may be fitted to

the data of such segments of these curves by the method of least squares so that leaf length, L, may be expressed as an explicit function of time, t, as follows:

$$L = a + bt + ct^2$$

where a, b, and c are constants. Hence, leaf growth which has been defined as dL/dt may be expressed by the relation

$$dL/dt = b + 2ct.$$

It was indicated above that within any given irrigation cycle, moisture stress can be empirically expressed as a function of time. It follows then that leaf length may be correspondingly derived as a tentative function of stress. Figure 5 presents the data for leaf length in figure 3 plotted against

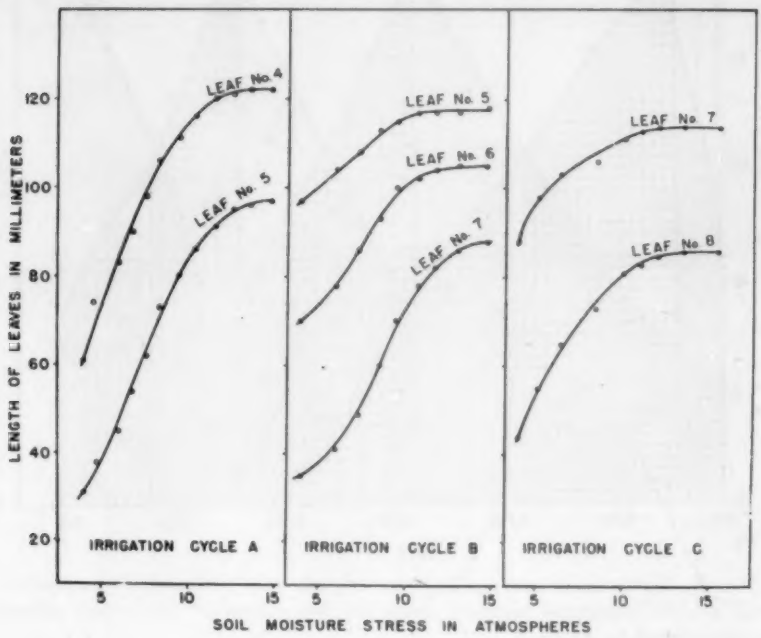

FIG. 5. Leaf elongation data of figure 3 plotted against the respective moisture stress data of figure 4.

corresponding observations for moisture stress shown in figure 4. These relationships are also essentially parabolic and could be expressed as

$$L = a' + b'S + c'S^2$$

where a′, b′, and c′ are constants determining the locus of a given curve.

Since leaf elongation tends to cease at the higher values of stress, it would be helpful to ascertain the intensity of stress associated with cessation of growth. This could be approximated by visual observation on figure 5, but personal bias would not be involved if this intensity of stress were arrived at mathematically from the empirical equation for the length of a given leaf as a function of stress during a given irrigation cycle.

As leaf elongation approaches cessation, the parabolic curve for the relation approaches a maximum. That is, the point at which the parabola passes through a maximum will be the theoretical point at which leaf elongation stops. It is to be recalled that the derivative of a parabolic equation becomes zero when the curve passes through a maximum. On differentiating, the previous equation becomes

$$dL/dS = b' + 2c'S.$$

When growth ceases, i.e., when $dL/dt = 0$, it follows that leaf length becomes invariant with respect to stress. Thus, at this time, $dL/dS = 0$. Consequently, the stress corresponding to the cessation of growth may be found from the relation

$$S = -b'/2c'.$$

To illustrate the mathematical procedure, the figures involved in the reduction of a typical set of data are presented in table I.

TABLE I

OBSERVATIONS MADE ON THE FIFTH LEAF OF PLANT NO. 1 DURING IRRIGATION CYCLE A

TIME (t) AFTER IRRIGATION	LENGTH OF LEAF (L)			INTEGRATED MOISTURE STRESS (S)	
	MEASURED	CALCULATED FROM L = f(t)	CALCULATED FROM L = f(S)	CALCULATED FROM W	CALCULATED FROM S = f(t)
Days	*mm.*	*mm.*	*mm.*	*atm.*	*atm.*
3	45	43.7	43.9	6.0	5.8
4	54	54.6	53.8	6.8	6.8
5	62	64.2	63.7	7.7	7.7
6	73	72.6	73.1	8.7	8.7
7	80	79.8	79.5	9.5	9.7
8	86	85.8	85.6	10.4	10.7
9	91	90.6	91.6	11.6	11.6
10	95	94.1	94.8	12.6	12.6
11	96	96.4	96.5	13.6	13.6
12	97	97.5	96.4	14.8	14.6

By the method of least squares, the empirical relation between L and t for these data was found to be:

$$L = 3.8 + 15.13\ t - 0.610\ t^2.$$

Hence,

$$dL/dt = 15.13 - 1.22\ t.$$

When $dL/dt = 0$,

$$t = \frac{15.13}{1.22} = 12.4 \text{ days},$$

the calculated time at which leaf elongation stopped. The data in table I also gave the following empirical relation:

$$S = 2.86 + 0.975\ t.$$

Thus, when leaf growth theoretically ceased on the 12.4th day,

$$S = 2.86 + 0.975 \times 12.4 = 14.9 \text{ atmospheres.}$$

As indicated above, it is not necessary, mathematically, to evaluate both L and S as functions of t before developing the relation between leaf length and stress. It may be shown from the data in table I that

$$L = -62.26 + 22.46S - 0.793\,S^2$$

and that

$$dL/dS = 22.46 - 1.586\,S.$$

Hence, when $dL/dS = 0$,

$$S = \frac{22.46}{1.586} = 14.2 \text{ atmospheres}$$

at the time when leaf elongation theoretically stopped.

The calculated values for L as a function of t in table I are slightly different from those calculated from the equation for L as a function of S. Of especial importance is the evidence that the derived curve, $L = f(t)$, had not passed through its maximum as of the 12th day, whereas the derived curve, $L = f(S)$, had passed through its maximum at this respective time. It is apparent that physiological logic dictates that growth be evaluated as a function of time, with the stress limiting to growth being calculated for the time when $dL/dt = 0$. Since the mathematical labor is considerably reduced by deriving L directly as a function of S, the values reported herein were calculated by the latter method. The difference between the limiting stress values calculated by the two different methods is well within experimental error.

It should be noted that some of the leaf elongation curves in figures 4 and 5 are somewhat sigmoid. Therefore, in deriving the constants for a given parabolic equation it was advisable to eliminate those values below the inflection point of an apparent sigmoid relationship.

Figure 6 shows the rate of change of leaf length as related to stress, i.e.,

$$dL/dS = b' + 2c'S.$$

The maximum rate of change of leaf length with respect to stress occurs, theoretically, when the stress is zero and is equal to the constant b′ in the equation. This value varied from 5.75 for the seventh leaf during irrigation cycle C to 24.6 for the seventh leaf during irrigation cycle B. However, it is apparent from the curves in figure 6 that for those leaves for which b′ is large, the rate of decrease of dL/dS with respect to S is also large. Consequently, the regression lines intercept the abscissa for moisture stress within a narrow range of values. This is shown more fully in table II, which presents the growth-limiting values as calculated for all cases.

There are minor variations in the values of moisture stress limiting to growth among the leaves of a given plant. There are similar variations among observations for respective leaves on different plants. Nevertheless, it is indeed remarkable that these values average close to 15 atmospheres. When one takes into account the inherent errors in the method-

ology, it is surprising that variation of the individual observations around the mean were not greater.

It is pertinent to note that RICHARDS and WEAVER (4) state that the growth of most crop plants stops before the soil moisture tension reaches 15 atmospheres and the condition of permanent wilting is attained. They have shown that for most soils the fifteen-atmosphere percentage lies within the wilting range of soil moisture.

FURR and TAYLOR (3) made daily measurements on the size of lemon fruits as the soil under the tree progressively dried out. They used the

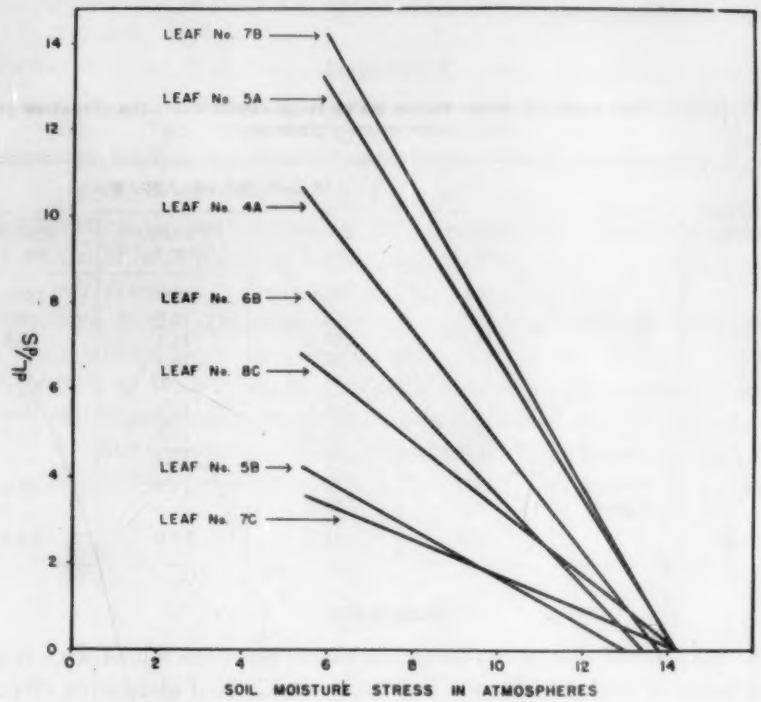

FIG. 6. The rate of change of leaf length as related to moisture stress.

apparent rate of increase in volume of the fruits as an index of turgescence within the tree. Their observations indicated that as the soil moisture is depleted a turgor deficit arises within the tree before the first visible sign of wilting appears. That is, growth was checked by water deficits within the plant, induced by increasing soil moisture stress. THUT and LOOMIS (6) reach this same conclusion from their studies involving consideration of many environmental factors in addition to supply of available soil moisture.

The data here presented are in substantiation of the theoretical considerations of BROYER (2) on the movement of water into plants. Of especial interest is his diagrammatic representation (see p. 20) showing the relationship between the various osmotic and hydrostatic forces and the relative

volumes of the cells subject to these forces. Relative volume is shown to be related to the hydrostatic specific free energy of the internal phase, i.e., to turgor pressure. In an absorbing cell of the root, this hydrostatic specific free energy is equal to the sum of the influx specific free energies of the cells diminished by the specific free energy of the soil moisture. In other words, enlargement of plant tissues—growth—is a direct function of turgescence which in turn is partially conditioned upon the force with which water is withheld from the plant. It follows, therefore, that the observations on leaf elongation herein presented are in line with BROYER's theoretical treatment of plant-water relations.

TABLE II

SOIL MOISTURE STRESS VALUES FOUND TO BE ASSOCIATED WITH CESSATION OF GROWTH OF COTTON LEAVES

IRRIGATION CYCLE	LEAF NO.	$S = -b'/2c'$ when $dG/dS = 0$			
		PLANT NO. 1	PLANT NO. 2	PLANT NO. 3	PLANT NO. 4
		atm.	*atm.*	*atm.*	*atm.*
1st	Fourth	13.8	.....	15.6	13.7
	Fifth	14.2	15.9	14.1	14.6
	Sixth	.....	16.7	18.1	.....
2nd	Fourth	.....	.....	.....	14.1
	Fifth	13.0	15.5	14.1	16.0
	Sixth	13.2	14.8	15.0	13.2
	Seventh	14.1	16.7	15.4	.....
3rd	Seventh	14.2	13.1	12.9	15.0
	Eighth	14.3	13.1	.....	.....
Average		13.8	15.1	15.0	14.4

Summary

The rate of leaf elongation on cotton plants was ascertained with respect to increasing intensity of the soil moisture stress. Leaf elongation virtually ceased at the higher intensities of induced stress and resumed on alleviation of the stress by irrigation. During a given irrigation cycle, elongation was expressed empirically as a second degree function of soil moisture stress. This functional relationship was characterized by an approach to a maximum at the theoretical time at which growth ceased. Since the derivative becomes zero at a maximum, this value is used in solving the differentiated equations for the intensity of soil moisture stress limiting to leaf elongation by using the empirically derived constants for the functional relationship between leaf length and moisture stress. This procedure gave values consistently close to 15 atmospheres for the moisture stress inducing leaf-growth cessation for (a) different irrigation cycles during the growth of a given leaf; (b) different leaves on the same plant; and, (c) leaves on different plants.

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GASEOUS LOSSES OF NITROGEN FROM GREEN PLANTS. I. STUDIES WITH CHLORELLA AND LEMNA

F. E. Allison, Katharine S. Love, L. A. Pinck and V. L. Gaddy

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Comparatively few experiments have been reported where plants have been tested in the absence of microörganisms for the liberation of gaseous nitrogen either from the tissues or from the substrate. There is some circumstantial evidence to indicate that losses sometimes occur. Such evidence is based in part on the frequent failure in the various types of experiments to account for all of the nitrogen supplied to growing plants. Furthermore, it is known that plants synthesize certain nitrogenous compounds, such as ammonia, HCN (**3**) and nicotine, that might under some conditions be volatilized from the tissues or from water that is exuded from plants during cool nights. Circumstantial evidence based upon low nitrogen recoveries from crops growing in normal unsterilized soil is, of course, of little value so far as indicating the extent to which higher plants are responsible for the losses. Many bacteria produce ammonia and other forms of volatile nitrogen, including N_2 gas. Some of the possibilities are discussed by Wilson (**5**) and need not be reviewed here.

The experimental difficulties involved in testing green plants apart from microörganisms for nitrogen evolution are obviously great. Therefore, the few studies of this type reported have been almost entirely with (a) small plants such as algae, with (b) germinating seeds or seedlings, and with (c) parts of plants that can be sterilized and maintained for short periods in nutrient cultures. Examples of experiments of type (a) and (c) are those of Pearsall and Billimoria (**4**), where large losses of nitrogen were observed with *Chlorella* and with *Narcissus* leaves floated on nutrient solutions. Davidson's (**2**) experiments with wheat and cowpeas, where no losses were observed, illustrate the (b) type of experimentation.

The results of Pearsall and Billimoria are so striking that a brief summary of their experiments is profitable. In their comparatively few experiments with Chlorella, the organisms were grown in a heavily buffered phosphate medium (pH6) containing 1 per cent. glucose and approximately 13 mg. of nitrogen as $NaNO_3$ per 50 ml. of nutrient solution. After incubation for 28 days in darkness, nearly two-thirds of the added nitrogen had disappeared; after 11 days in the light the loss was 6 per cent.

In their more extensive work with Narcissus, sterilized leaves were floated on nutrient solutions containing various forms of nitrogen, glucose, and phosphates to buffer the solution at pH 5.4. Different portions of old and young leaves were incubated in the culture media in both light and darkness. No losses were observed where organic sources of nitrogen were

used. Losses, sometimes exceeding 50 per cent., were found in both light and dark cultures where inorganic sources of nitrogen were supplied.

In initiating studies on gaseous losses of nitrogen from plants and soils, it seemed advisable to examine them further to determine if losses of the magnitude of those reported by PEARSALL and BILLIMORIA are common. The present paper reports the results of studies with Chlorella and Lemna; the second paper of the series gives the data where the leaves of Narcissus and other plants were floated on nutrient solutions in the light and in the dark.

Experimental methods

The organisms used included two strains of *Chlorella vulgaris,* one (strain A) obtained from Dr. E. S. Johnston, Smithsonian Institution, and the other (strain B) from Dr. M. A. Brannon, University of Florida; *Chlorella pyrenoidosa* from Dr. Florence E. Meier, Smithsonian Institution; and *Lemna minor* from Dr. R. A. Steinberg of this Bureau.

The nutrient medium used for growing the algae, unless otherwise stated, had the following composition per liter:

KH_2PO_4	10.0 gm.
$MgSO_4$	0.2 "
$CaSO_4$	0.1 "
Sodium citrate	0.2 "
Sucrose	10.0 "
$FeSO_4$	1.5 drops of saturated solution

In addition, ARNON's (1) A–5 solution of minor elements to give a final concentration in p.p.m. of Mn 0.5, B 0.5, Zn 0.05, Cu 0.02 and Mo 0.01, was added. The form and concentration of the nitrogen compounds, which varied, is given below. The media used for growing *Lemna minor* are also given in connection with the subsequent discussion.

All cultures were maintained either in a dark incubator at a constant temperature of 28° C., or in a thermostatted room under a bank of fluorescent lamps at a temperature of 23° C. The culture vessels were usually 250 ml. Erlenmeyer flasks containing 50 ml. of the medium. In one experiment (table IV), large test tubes were used. Analyses of the organisms plus medium for total nitrogen were made by the Davisson-Parsons method if nitrate was present, or by the Gunning method if it was absent. The cultures were not dried prior to analysis.

Experimental results

PRELIMINARY EXPERIMENTS WITH CHLORELLA GROWN IN THE LIGHT AND DARK ON VARIOUS MEDIA.—Several experiments were conducted for the purpose of determining the best medium to use. Most of those recommended by previous workers were found unsatisfactory for the present studies

where it was desired to compare nitrogen sources. Where only 0.1 to 0.2 per cent. phosphate was added the pH tended to vary widely from near 4.0 for some nitrogen sources to near 9.0 for others.

Comparisons were also made between growth on sucrose and glucose. In some media, the latter energy source breaks down during sterilization to yield toxic substances; hence, sucrose is preferable if utilized. All three cultures of Chlorella grew equally well on the two sugars in both light and darkness, except that *Chlorella vulgaris* (strain B) usually grew rather poorly on sucrose in the dark.

Nitrogen analyses made on a number of the cultures that grew best showed no significant losses of nitrogen. Table I gives the analytical results

TABLE I

THE EFFECT OF *Chlorella vulgaris* (STRAIN A) ON THE NITROGEN CONTENT OF MEDIA AFTER INCUBATION IN THE LIGHT AND DARK*

NITROGEN SOURCE	pH AT END OF EXPERIMENT	NITROGEN CONTENT OF CELLS	ORIGINAL TOTAL N	FINAL TOTAL N	NITROGEN LOSS OR GAIN
		mg.	*mg.*	*mg.*	*mg.*
			In light—12 days		
Potassium nitrate	7.9	5.91	12.05	12.40	+0.35
Alanine	4.5	4.88	12.88	12.70	−0.18
Asparagine†	6.3	5.37	11.27	11.06	−0.21
Ammonia sulfate†	4.2	10.54	12.79	12.67	−0.12
Urea	5.0	11.46	12.46	12.15	−0.31
Ammonium nitrate†	6.6	11.97	12.29	12.26	−0.03
			In dark—40 days		
Potassium nitrate	6.2	2.02	12.05	12.32	+0.27
Alanine	4.5	1.59	12.88	12.77	−0.11
Asparagine	4.4	1.22	11.10	11.44	+0.34
Asparagine†	5.7	3.59	11.27	11.41	+0.14
Ammonium sulfate†	5.5	5.69	12.79	12.61	−0.18
Urea	5.3	5.13	12.46	12.35	−0.11
Ammonium nitrate†	5.3	5.20	12.29	12.36	+0.07

* All values given are the averages of duplicate determinations.
† 0.1% $CaCO_3$ added.

for one such experiment. The medium in this case was the same as given above except for the use of 1.225 grams of KH_2PO_4 per liter, instead of 10 grams, and the omission of the sodium citrate. In a few cases sterile $CaCO_3$ was added separately at the rate of one gram per liter. Nitrogen in the various forms was added in such amount as to supply near 13 mgm. of nitrogen per 50 ml. of culture.

The data in table I show no significant losses of nitrogen from any of the six sources of nitrogen used in Chlorella cultures grown in either the light or dark. Growth in the dark was considerably less than in the light, even though the growth period was more than three times as long. The nitrogen content of the cells, shown in column 3, is of course a measure of growth. In the low phosphate medium used it will be observed that the pH of the culture varied widely with the nitrogen source.

Since the medium used in the above experiment did not adequately control the pH, experiments were conducted to determine the maximum concentration of phosphate that the organisms would tolerate at a pH of near 6.0 without showing retarded growth. This concentration was found to be 10 grams KH_2PO_4 per liter for *Chlorella vulgaris* (strain A), and hence was used in subsequent experiments.

THE GROWTH OF CHLORELLA ON VARIOUS NITROGEN SOURCES IN THE LIGHT AND DARK.—In table II are given the results of analyses of Chlorella

TABLE II

THE NITROGEN CONTENT OF CULTURES OF *Chlorella vulgaris* (STRAIN A) GROWN ON VARIOUS NITROGEN SOURCES IN THE LIGHT AND DARK*

NITROGEN SOURCE	AGE OF CULTURES DAYS	pH AT END OF EXPERIMENT	ORIGINAL TOTAL N	FINAL TOTAL N	NITROGEN LOSS OR GAIN
			mg.	*mg.*	*mg.*
		In light			
Sodium nitrate	13	6.0	11.77	12.04	+0.27
Ammonium nitrate‡	13	4.2	11.95	10.23	−1.72
Ammonium sulfate	13	3.1	12.83	12.08	−0.75
Ammonium sulfate†	13	4.6	12.63	12.23	−0.40
Alanine	13	5.3	12.85	12.55	−0.30
Asparagine	13	5.1	11.11	11.09	−0.02
Sodium nitrate	27	6.4	11.77	12.22	+0.45
Ammonium sulfate†	27	4.7	12.63	12.32	−0.31
Alanine	27	5.3	12.85	12.67	−0.18
Asparagine	27	5.4	11.11	10.85	−0.26
		In dark			
Sodium nitrate	31	5.3	11.77	11.74	−0.03
Ammonium nitrate	31	4.4	11.95	10.83	−1.12
Ammonium sulfate	31	3.8	12.83	12.62	−0.21
Ammonium sulfate†	31	5.5	12.63	12.28	−0.35
Alanine	31	4.9	12.85	12.67	−0.18
Asparagine	31	4.8	11.11	10.86	−0.25
Sodium nitrate	66	.....	11.77	11.68	−0.09
Ammonium nitrate‡	66	.....	11.95	11.02	−0.93
Ammonium sulfate‡	66	.....	12.83	12.58	−0.25
Ammonium sulfate†	66	.....	12.63	12.24	−0.39
Alanine	66	.....	12.85	12.76	−0.09
Asparagine	66	.....	11.11	11.06	−0.05

* All values given are the averages of duplicate determinations.
† 0.1% $CaCO_3$ added.
‡ These cultures were dead or nearly so when analyzed. The corresponding cultures grown in the light for 27 days were dead and hence were not analyzed.

cultures grown on five sources of nitrogen in the light and dark in the high phosphate medium. Even the one per cent. phosphate buffer was inadequate to keep the medium at pH 6.

There was a loss of approximately 10 per cent. of the added nitrogen where ammonium nitrate was used. This loss occurred under rather abnormal conditions due to the marked decrease in pH to the point where the organism was killed. This increase in acidity was due to the preferential assimilation of ammonia by the algae, and occurred so quickly that only a

comparatively small amount of growth occurred before the organisms began to die. There is a slight indication of a similar but smaller loss of nitrogen from ammonia sulfate under similar conditions, but the data are inadequate to establish this point. Where the other sources of nitrogen (sodium nitrate, alanine, and asparagine) were used no significant losses occurred, either in the light or in the dark.

THE GROWTH OF CHLORELLA ON VARIOUS NITROGEN SOURCES AND COMBINATIONS IN THE LIGHT AND DARK.—Additional analytical data, given in table

TABLE III

THE NITROGEN CONTENT OF CULTURES OF *Chlorella* GROWN ON VARIOUS NITROGEN SOURCES IN THE LIGHT AND DARK*

NITROGEN SOURCE	ORGANISM	ORIGINAL TOTAL N	FINAL TOTAL N	NITROGEN LOSS OR GAIN
		mg.	*mg.*	*mg.*
	In light—18 days			
Sodium nitrate	*C. vulgaris*, Str. A	12.01	11.86	−0.15
Ammonium nitrate†	"	12.26	12.37	+0.11
Ammonium sulfate†	"	12.75	12.48	−0.27
Alanine	"	12.43	12.46	+0.03
Sodium nitrate + ammonium sulfate†	"	12.19	12.53	+0.34
Sodium nitrate + alanine	"	12.33	12.43	+0.10
Ammonium nitrate	"	12.25	10.78	−1.47
Sodium nitrate	*C. vulgaris*, Str. B	12.01	11.31	−0.70
Sodium nitrate + alanine	"	12.33	11.82	−0.51
Sodium nitrate	*C. pyrenoidosa*	12.01	11.90	−0.11
Sodium nitrate + alanine	"	12.33	12.27	−0.06
	In dark—52 days			
Sodium nitrate	*C. vulgaris*, Str. A	12.01	11.99	−0.02
Ammonium nitrate†	"	12.26	12.44	+0.18
Ammonium sulfate†	"	12.75	12.74	−0.01
Alanine	"	12.43	12.87	+0.44
Sodium nitrate + ammonium sulfate†	"	12.19	12.50	+0.31
Sodium nitrate + alanine	"	12.33	12.70	+0.37
Ammonium nitrate	"	12.25	11.72	−0.53
Sodium nitrate	*C. pyrenoidosa*	12.01	12.31	+0.30
Sodium nitrate + alanine	"	12.33	12.33	0.00

* All values given are the averages of duplicate determinations.
† 0.1% $CaCO_3$ added.

III, agree with those in the two previous tables in showing no significant nitrogen losses with any of the nitrogen sources, except possibly ammonium nitrate. The maximum loss in this case was near 12 per cent. As in the previous experiment, this loss occurred under such acid conditions that the cultures grew only a few days before becoming very unhealthy. Where $CaCO_3$ at the rate of 0.1 per cent. was added to counteract the acidity, the growth was excellent and no loss of nitrogen occurred. Where *Chlorella vulgaris* (strain B) was grown, there was an apparent slight loss of nitrogen from both sodium nitrate and sodium nitrate plus alanine, but the values are undoubtedly within experimental error.

Much emphasis has been given in the literature (5) to the possible loss of nitrogen by the Van Slyke reaction involving the interaction of nitrites with ammonia or amines to liberate nitrogen gas from each. Nitrite is of course formed by green plants and many microörganisms as a result of reducing nitrates. In the present experiment mixtures of nitrates with either alanine or ammonia were supplied in order to test for the occurrence

TABLE IV

THE NITROGEN CONTENT OF CULTURES OF *Chlorella* GROWN IN THE DARK ON VARIOUS NITROGEN SOURCES UNDER CONDITIONS OF GOOD AND POOR AERATION*

NITROGEN SOURCE	ORGANISM	AERATION	ORIGINAL TOTAL N	FINAL TOTAL N	NITROGEN LOSS OR GAIN
			mg.	*mg.*	*mg.*
		52 days			
Sodium nitrate	*C. vulgaris*, Str. A	Good	12.01	12.31	+0.30
" "	"	Poor	12.01	12.56	+0.55
Ammonium nitrate†	"	Good	12.26	12.44	+0.18
" "	"	Poor	12.26	10.94	−1.32
Alanine	"	Good	12.43	12.83	+0.40
"	"	Poor	12.43	13.05	+0.62
Sodium nitrate + alanine		Good	12.33	12.70	+0.37
" " "	"	Poor	12.33	12.42	+0.19
Ammonium nitrate	"	Good	12.25	11.74	−0.51
" "	"	Poor	12.25	12.15	−0.10
Sodium nitrate	*C. vulgaris*, Str. B	Poor	12.01	11.59	−0.42
" "	*C. pyrenoidosa*	Good	12.01	12.29	+0.28
" "	"	Poor	12.01	11.31	−0.70
		102 days			
Sodium nitrate	*C. vulgaris*, Str. A	Good	12.01	12.10	+0.09
" "	"	Poor	12.01	12.52	+0.51
Ammonium nitrate†	"	Good	12.26	11.41	−0.85
" "	"	Poor	12.26	12.14	−0.12
Alanine	"	Good	12.43	12.67	+0.24
"	"	Poor	12.43	12.74	+0.31
Sodium nitrate + alanine		Good	12.33	12.34	+0.01
" " "	"	Poor	12.33	12.15	−0.18
Ammonium nitrate	"	Good	12.25	10.50	−1.75
" "	"	Poor	12.25	11.50	−0.75
Sodium nitrate	*C. vulgaris*, Str. B	Poor	12.01	11.86	−0.15
" "	*C. pyrenoidosa*	Good	12.01	11.60	−0.41
" "	"	Poor	12.01	12.04	+0.03

* All values given are the averages of duplicate determinations.
† 0.1% $CaCO_3$ added.

of the Van Slyke reaction. There was no evidence for this reaction except possibly in the case of ammonium nitrate, where normal growth did not occur.

THE GROWTH OF CHLORELLA IN THE DARK ON VARIOUS NITROGEN SOURCES UNDER CONDITIONS OF GOOD AND POOR AERATION.—It seemed probable that in the presence of limited oxygen, conditions would be more favorable for nitrite formation from nitrates and for loss of nitrogen, either through reaction with ammonia or amines or through volatilization of oxides of nitrogen. The experiment reported in table IV was therefore conducted.

Good aeration was provided by adding the usual 50 ml. of medium to cotton-plugged Erlenmeyer flasks of 250 ml. capacity. Poor aeration was provided by using cotton-plugged test tubes having a diameter of $\frac{1}{8}$ inch. To each of these was added 50 ml. of the medium to give a depth of approximately 5.5 inches.

The data show very little, if any, effect of aeration on nitrogen losses. The usual losses of about 10 per cent. of the nitrogen added as ammonium nitrate were obtained, but these losses were not always greater where the aeration was poor. The slight losses sometimes observed where sodium nitrate was present were no greater than the gains in other such cultures.

THE GROWTH OF LEMNA ON DIFFERENT NITROGEN SOURCES.—In initiating nitrogen loss studies it was hoped that *Lemna minor* or *Spirodella polyorhiza* could be used in much of the work. Considerable difficulty was, however, experienced in growing these organisms due largely to their sensitiveness to the proper kinds and concentrations of minor elements, and to their inability to tolerate acidity. The minor element difficulty was solved by the addition of Arnon's A–5 solution to either one of the three media given in table V. The acidity factor was not overcome because these organisms require a pH of near 6 or above and cannot tolerate high concentrations of phosphate buffer. Numerous attempts to use various sources of nitrogen, particularly ammonium salts, resulted in no growth. Excellent growth was, however, obtained in several experiments using either asparagine, sodium nitrate, or potassium nitrate. Table V gives the analytical results for a

TABLE V

THE NITROGEN CONTENT OF CULTURES OF *Lemna minor* GROWN ON ASPARAGINE AND NITRATES*

NITROGEN SOURCE	MEDIUM†	ORIGINAL TOTAL N	FINAL TOTAL N	NITROGEN LOSS OR GAIN
		mg.	*mg.*	*mg.*
Sodium nitrate	A	12.20	12.55	+0.35
Potassium nitrate	B	5.35	5.40	+0.05
Asparagine	C	4.70	4.80	+0.10

* All values given are the averages of duplicate determinations.

† The media used had the following compositions in terms of grams per liter:

	A	B	C
KH_2PO_4	1.225	—	0.100
$CaH_4(PO_4)_2 \cdot H_2O$	—	0.100	1.000
$MgSO_4$	1.204	0.250	0.250
$CaSO_4 \cdot 2H_2O$	0.100	—	—
$FeCl_3$	0.002	0.002	0.002
Sucrose	10.0	10.0	10.0
$NaNO_3$	1.594	—	—
KNO_3	—	0.800	—
Asparagine	—	—	0.524

Arnon's A–5 solution was supplied to all media.

typical experiment. Lemna did not, under the conditions, cause a loss of gaseous nitrogen from either nitrates or asparagine.

Discussion

Experiments done under conditions similar to those of PEARSALL and BILLIMORIA (**4**) fail to support their findings that *Chlorella* may cause large losses of nitrogen. In their experiments, where sodium nitrate was supplied to cultures kept in the dark, as much as two-thirds of the added nitrogen was not recovered. In the present experiments the losses under similar conditions were within experimental error.

The only barely significant loss observed was a decrease of 0 to 14 per cent. of the nitrogen added as ammonium nitrate. This loss occurred under conditions of limited growth due to a rapid increase in pH to the point (about pH 4) where the organisms died. Death usually occurred before one-fifth of the total nitrogen was assimilated. The exact cause of the nitrogen loss from such dying cultures has not yet been established. The interaction of nitrites, formed from nitrate, with ammonia is one possibility. Volatilization of oxides of nitrogen from the unstable nitrous acid, if formed, is another possibility. Under more normal conditions of pH no loss of nitrogen occurred.

Summary

1. The results of experiments conducted chiefly with *Chlorella vulgaris*, and also with *Chlorella pyrenoidosa* and *Lemna minor*, to determine whether these plants bring about gaseous losses of nitrogen from various nitrogen sources, are reported. In the case of Chlorella the cultures were grown both in the light and dark for varying periods of time.

2. No significant loss of nitrogen was observed under any conditions where the following sources of nitrogen were used; potassium nitrate, sodium nitrate, ammonium sulfate, alanine, asparagine and urea. Losses were likewise not observed where either ammonium sulfate or alanine was used together with sodium nitrate. In such mixtures the possibility exists for a reaction of nitrite, formed from nitrates, with the ammonia or amino nitrogen to liberate nitrogen gas from each.

3. Losses of nitrogen amounting to as much as 14 per cent. of that added, but averaging only 5 per cent., were observed where ammonium nitrate was added. These losses occurred in *Chlorella* cultures that were dying due to the marked increase in acidity brought about by the preferential use of ammonia in its growth processes.

DIVISION OF SOIL MANAGEMENT AND IRRIGATION
BUREAU OF PLANT INDUSTRY, SOILS AND AGRICULTURAL ENGINEERING
AGRICULTURAL RESEARCH ADMINISTRATION
U. S. DEPARTMENT OF AGRICULTURE
BELTSVILLE, MARYLAND

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THE ROLE OF ACTIVE WATER ABSORPTION IN AUXIN-INDUCED WATER UPTAKE BY AERATED POTATO DISCS

J. LEVITT

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In recent years, the concept of active water absorption has been utilized to explain a variety of experimental results. LEVITT (**2**) has shown that some of these explanations are thermodynamically impossible and based on incorrect interpretations. The auxin-induced water uptake by potato discs has also been attributed to active water absorption [REINDERS (**3**), COMMONER *et al.* (**5**)]. In favor of this interpretation is REINDERS' discovery that the auxin usually very markedly increases the respiratory loss of dry matter (**3, 4**). However, she also obtained the opposite result under certain cond tions. VAN OVERBEEK (**7**) concluded that the evidence available is insufficient to permit a choice between active water absorption, reduced wall pressure, or both. Obviously, both the concept of water absorption and the mechanism of auxin action would be better understood if this problem were cleared up.

As VAN OVERBEEK (7) has pointed out, since the auxin-induced water uptake can occur from distilled water, it is not associated with active salt absorption. The only active absorption possible under these conditions is that of water. By definition, active absorption involves uptake against a diffusion gradient. The diffusion pressure of the water in the cell must therefore be *greater* than that of the almost pure water outside the cell—i.e., the D.P.D. must have a negative value. This can be true only if the turgor pressure (or wall pressure) is increased to a value greater than the osmotic pressure of the cell. But the absorption of water by the cell will be associated with an increased T.P. (or W.P.) only if the wall has not previously reached its elastic limit. Thus an elastic (reversible) stretch involves an increase in T.P. and W.P., a plastic (irreversible) stretch, does not.

Consequently, from the point of view of the kind of wall extension involved, there are two possible modes of active water absorption. (1) The absorption may occur against a diffusion gradient accompanied by an elastic stretch of the cell wall, both being reversible. Energy must be continually expended to retain this actively absorbed water in the cell. (2) The absorption may at first occur against a diffusion gradient, accompanied by a temporary elastic stretch of the wall. This elastic stretch is soon converted into a permanent set—i.e., it becomes a plastic stretch. The increased wall pressure simultaneously disappears, as does the negative diffusion gradient, and the actively absorbed water is now retained in diffusion equilibrium with the surrounding water. No energy need be expended to retain this water but some must be used initially to drive it into the cell.

The purpose of this investigation is to determine whether or not either one of these modes of active water absorption can explain the auxin-induced water uptake by potato tissue.

Methods

Though none of the other investigators consider it necessary, VAN OVERBEEK (7) points out that it is desirable to conduct the aeration of the potato discs under aseptic conditions, and in order to accomplish this, a somewhat simplified technique was adopted in this investigation. A scrubbed tuber (Russet variety) was wetted with alcohol and flamed. It was then peeled and sliced (2–4 mm. thick), the slices were washed with four changes of distilled water at 0–2° C. (using a total of about 2 liters), blotted with cotton gauze, and a uniform number (four or five slices) transferred to wide-mouthed weighing bottles. The weighing bottles were weighed, emptied into the prepared jars of distilled water and indole acetic acid (concentration 10^{-5}) respectively, then aerated continuously with aquarium or Pyrex aerators. The amount of liquid used was at least ten times the fresh weight of the slices. All these operations were, of course, performed under aseptic conditions. In some of the experiments, one weighing bottle of slices was kept for determinations of moisture content. Hand-slicing reduced the uniformity of the samples as compared with microtome slicing, and necessitated the use of larger and thicker slices; but it simplified the problem of maintaining aseptic conditions. Since the same number of slices was used in water as in indole acetic acid, the comparative average thickness of the slices could be judged by the fresh weights of the two samples. When the two weights differed excessively, the samples were discarded.

Even with this technique, aseptic conditions were not at first obtained. When, however, brass jar covers were used instead of cotton batting (the covers were greased and padded with non-absorbent cotton to prevent contamination with copper), asepsis was usually obtained. Since all the results in the literature, except van Overbeek's, were obtained without asepsis, some results with slight infection have been included for comparison.

At the end of the aeration period, whenever changes in dry weight were to be determined, the discs were again wiped dry, weighed, then freeze-dried over Al_2O_3 [LEVITT (1)]. They were finally transferred to desiccators containing $Mg(ClO_4)_2$ and evacuated. From time to time they were weighed and reevacuated until constant weights were obtained (after 10–14 days).

Theoretical considerations and results

As indicated above, there are two possible modes of active water absorption, depending on whether an initial elastic stretch of the cell wall is maintained, or whether it is converted into a plastic stretch. That the first mode is not the correct one is indicated by the fact that potato strips placed in distilled water stretch well beyond the elastic limit of their cell walls in a

period of four hours [LEVITT (**2**)]. Four lines of experimental evidence may be obtained to prove this more conclusively. a) A completely reversible (elastic) increasing stretch of the cell wall would be accompanied by an increasing W.P. and therefore less and less water would be actively absorbed and a steady state would soon be reached. If the stretch were converted into a plastic one (according to mode 2), the active absorption might be expected to continue indefinitely at a rate dependent on the balance between the decreasing O.P. of the cell, the probably decreasing wall pressure, and the energy released. REINDERS (**3, 4**) showed that the uptake does continue, at least for a period of as long as eight days. b) If mode 1 were correct, energy would have to be continuously expended to retain the actively absorbed water. This should be measurable. c) Anything that reduced this energy expenditure (e.g., transfer to a low temperature, or

TABLE I

EFFECT OF TRANSFER TO LOW TEMPERATURE ON WATER UPTAKE BY AERATED POTATO SLICES.

FIRST AERATION PERIOD	TEMP.	WATER UPTAKE (% OF ORIG. FRESH WT.)		SECOND AERATION PERIOD	TEMP.	WATER UPTAKE (% OF ORIG. FRESH WT.)	
hours	°C.	*in water*	*in IAA*	*hours*	°C.	*in water*	*in IAA*
48	25	50.8*	52.1*	24	0–2	0.82	0.41
72	24	50.2	53.0*	24	0–2	0.37	0.35
72	24	45.9	47.9	24	0–2	0.67	0.76
72	24	42.0*	48.2*	26	0–2	0.40	0.47
72	22½–24	40.8	45.3	24	0–2	0.11	1.47
72	22½–24	42.6	46.6	24	0–2	0.24	0.36

* Signifies slight infection, others aseptic

presence of respiration inhibitors) would be followed by a loss of actively absorbed water. d) A permanent elastic stretch such as would occur in mode 1 should be measurable. Transfer to a hypertonic solution should cause a greater shrinkage in the auxin-treated than in the control water-treated.

Any one of the above lines of evidence should be capable of proving whether or not mode 1 is possible. In view of its simplicity, experiment c was attempted. Potato discs were allowed to absorb water for 2–3 days at room temperature from distilled water and indole acetic acid (conc. of 10^{-5}), respectively. The amount of water absorbed was determined by weighing, and the aeration was then continued at 0–2° C. for 24 hours. Table I shows that at this low temperature there was no loss of absorbed water but, on the contrary, an actual increase. With one exception, this increase was at least as great in the indole acetic acid as in the distilled water. Such a result would be impossible if energy were required to retain the auxin-induced water uptake. Consequently, the second mode of active water absorption appears to be the only one possible. Even this mode seems unlikely in view of the greater average uptake in indole

acetic acid at 0–2° C. But the results are not conclusive on this score. The problem was therefore next attacked from the point of view of the energy relations involved.

If the second mode of active water absorption is correct, energy will be needed to obtain the auxin-induced water uptake but not to retain it. The energy needed can be calculated from the work done in moving the water against the diffusion gradient:

$$\text{work done} = p \Delta v \quad (1)$$

where p is the net diffusion pressure against which the water must be moved and Δv is the increase in cell volume due to the active absorption of water. Both of these values can be calculated from measurements of the absorption of water by duplicate sets of potato discs in pure water and in indole acetic acid. p must be the excess of the D.P. of the water in the actively absorbing cells over that of the surrounding water:

$$p = D.P._i - D.P._o \quad (2)$$

where $D.P._i$ = diffusion pressure of water in potato discs in indole acetic acid and $D.P._o$ = diffusion pressure of surrounding water.

TABLE II

FREEZING POINT LOWERING (Δ) OF WATER AND INDOLE ACETIC ACID (10^{-5}) AFTER AERATING POTATO DISCS IN THEM FOR TWO TO FOUR DAYS

AERATION TIME	Δ WATER	Δ INDOLE ACETIC ACID
hours	°C	°C
47	0.025	0.015
95	0.015	0.015
71	0.030	0.020

Since we are interested in an increased energy consumption due to auxin-induced uptake, we can assume that the control discs (in water) are absorbing water only passively. Then:

$$D.P._o = D.P._c \quad (3)$$

where $D.P._c$ = diffusion pressure of the water in the control discs.

$$\text{Therefore } p = D.P._i - D.P._c \quad (4)$$

Equation (4) holds true only if $D.P._o$ is identical in the two cases. Since only 10 ppm. of indole acetic acid is used, the diffusion pressure of this solution may be considered equal to that of distilled water. However, it is conceivable that the indole acetic acid might either induce or reduce a diffusion of solutes from the tissues into the surrounding water. Consequently, freezing point determinations were made at the end of the experiment. There was no significant difference between the distilled water and the indole acetic acid (table II). This is in agreement with REINDERS (**4**), as she was unable to find any difference in conductivity. The assumption on which equation (4) is based is therefore valid.

But for any cells:

$$D.P. = D.P._w + W.P. - O.P. \quad (5)$$

where $D.P._w$ = diffusion pressure of pure water, W.P. = cell wall pressure, O.P. = osmotic pressure of cell sap. Therefore, from (4) and (5),

$$p = (D.P._w + W.P._i - O.P._i) - (D.P._w + W.P._c - O.P._c)$$
$$= (W.P._i - W.P._c) + (O.P._c - O.P._i) \quad (6)$$

$O.P._c$ and $O.P._i$ are easily obtained experimentally. $W.P._c$ and $W.P._i$ must be obtained indirectly.

If $O.P._c$ and $O.P._i$ are the values at the end of the experiment, then in order to calculate the work done, an average value from the beginning of the active uptake till the end must be used:

$$\text{av. } p = \tfrac{1}{2}\,[(W.P._i - W.P._c) + (O.P._c - O.P._i)] \quad (7)$$

Now $\Delta v = v_i - v_c$ where v_i = volume water absorbed by the potato discs in indole acetic acid, and v_c = volume absorbed by an equal quantity of potato discs in distilled water. Consequently,

$$p\,\Delta v = 0.5\,[(W.P._i - W.P._c) + (O.P._c - O.P._i)] \times (v_i - v_c)$$

If W.P. and O.P. are expressed in atms., v_i and v_c in ml. and the heat of combustion of glucose = 3.75 Kcal./gm., then glucose loss (gms.)

$$= 0.5\,[(W.P._i - W.P._c) + (O.P._c - O.P._i)]\;(v_i - v_c) \times \frac{1.013 \times 10^6}{4.182 \times 10^7 \times 3.75 \times 10^3}$$
$$= 3.23 \times 10^{-6}\,[(W.P._i - W.P._c) + (O.P._c - O.P._i)]\;(v_i - v_c) \quad (8)$$

TABLE III

CALCULATED VALUES FOR LOSS IN DRY WEIGHT REQUIRED TO SUPPLY THE ENERGY FOR THE ACTIVE ABSORPTION OF WATER BY 100 GRAMS OF POTATO SLICES HAVING AN ORIGINAL O.P. OF 10 ATMS.

$V_i - V_c$ (ML. OR GMS. WATER)	INCREASE IN FRESH WT.	O.P.	$2(O.P._c - O.P._i)$	LOSS IN DRY WEIGHT
	%	*atms.*	*atms.*	*microgm.*
0	40	6.7	.....	.....
5	45	6.4	0.6	9.7
10	50	6.15	1.1	35.5

Before proceeding with experimental results, it is well to find out whether this hypothetical glucose loss is measurable. We shall assume an original osmotic value of 10 atms. for the potato tissue, and an uptake of water in the aerated control discs equal to 40% of the original fresh weight. Furthermore, for a rough approximation we shall consider the increase in wall pressure in the indole acetic acid equal to the decrease in osmotic value. All these assumptions are close enough to the experimentally determined values to enable us to judge whether or not the quantity will be measurable.

Table III shows that an active, auxin-induced uptake of as high as 10 grams of water per 100 grams of tissue would require the respiratory loss of only some 35 micrograms of sugar. Small as this calculated value

is, the actual value is undoubtedly even less, if the second mode of active absorption is involved. The above calculations would hold (approximately) for the active absorption if the cell wall is stretched elastically. If, however, a small elastic stretch is soon followed by a permanent set, p would never attain a value of more than a small fraction of that used in the above calculations. Consequently, if mode two is the correct one, the energy requirements would be insignificant and indetectable, unless the portion utilized is a very minute fraction of the total increase in available respiratory energy—i.e., if the efficiency of utilization is extremely low. REINDERS (**4**), however, has reported very definite increases (as high as 100%) in respiratory loss accompanying auxin-induced water uptake. This was true over a period of eight days, yet during the first few days the respiratory loss was usually actually reduced by heteroauxin, though this was the period of greatest increase in water uptake. Reinders logically concluded that the auxin-induced water uptake was completely separate from the auxin-induced respiratory loss in dry matter.

TABLE IV

WATER UPTAKE AND LOSS IN DRY WEIGHT OF POTATO DISCS AERATED IN WATER AND IN INDOLE ACETIC ACID. (10^{-5}). THE FIRST FOUR SERIES WERE SLIGHTLY INFECTED, THE LAST THREE WERE ASEPTIC

DATE	AERATION TIME	ORIGINAL FRESH WT.		WATER UPTAKE (% INCREASE IN FR. WT.)		LOSS IN DRY WT. (% OF ORIGINAL DRY WT.)	
	hours	*water*	*IAA*	*water*	*IAA*	*water*	*IAA*
Sept. 9	47	57.105	62.120	30.0	30.4	2.5	1.25
Sept. 12	95	64.102	52.673	40.1	41.6	4.4	4.8
Sept. 17	71	36.820	41.838	39.9	43.6	6.3	3.3
Sept. 20	71	54.454	50.009	46.5	50.6	4.8	1.1
Oct. 31	96	19.988	16.922	36.5	48.6	11.9	10.7
Nov. 13	52	25.336	21.315	49.2	51.3	18.8	16.6
Dec. 2	48	34.039	33.409	55.6	52.2*	6.3	5.0

* Injury evident in one region which was soft and flaccid. Later tests indicated that this was due to the presence of copper in the water

The results obtained in this investigation agree, in the main, with Reinders. The indole acetic acid reduced the loss in dry weight over a period of 2–4 days (table IV). The experiments were repeated at different times of the year (table V). Of seven tests in April and May, five again showed a smaller loss in dry weight in the presence of IAA, but two showed the opposite change. Four tests made in June and July all showed a greater loss of dry weight in the IAA. Two of these absorbed less water than the controls. All six experiments that showed a greater loss of dry matter in IAA were conducted at temperatures of 25–29° C., as compared with temperatures of 21–24° C. for the other five. Similarly, the other seven tests in fall and winter (table IV) were conducted at this lower temperature.

These results agree completely with Reinders'. Though eight days'

TABLE V

WATER UPTAKE AND LOSS IN DRY WEIGHT OF POTATO DISCS AERATED IN WATER AND IN INDOLE ACETIC ACID (10^{-5})

DATE	AERATION TIME	ORIGINAL FRESH WT.		WATER UPTAKE (% INCREASE IN F.W.)		LOSS IN DRY WT. (% OF ORIGINAL DRY WT.)	
	hours	*water*	*IAA*	*water*	*IAA*	*water*	*IAA*
April 28	48	20.798	21.798	43.1	44.2	3.1	0.2
May 3	76	15.322	15.358	40.4	49.4	4.5	4.3
" 9	96	24.752	23.313	37.5	38.9	2.1	+1.3
" 14	70	*21.312	22.342	40.8	43.9	3.9	4.3
" 20	72	22.370	23.261	34.0	38.0	7.8	5.2
" 24	60	*21.574	24.499	23.2	26.3	7.9	5.6
" 27	72	24.956	24.230	35.8	38.3	0.0	3.3
June 27	72	19.897	16.354	46.1	41.6	13.4	16.3
" 27	144	21.734	22.684	55.6	52.3	9.6	14.7
July 4	72	*18.038	*20.050	37.1	40.5	5.8	6.7
" 16	144	*23.553	20.507	55.5	59.9	9.4	14.5

* Aseptic except where marked.

exposure to auxin at 21° C. (Reinders) and 3–4 days' exposure to 25–29° C. (Levitt) permits a greater loss of dry matter when auxin is present, 3–4 days' exposure of 21°–24° C. results in a considerably smaller loss of dry matter when auxin is present (Reinders, Levitt). And it is during this period of auxin-inhibited respiration that the auxin-induced water uptake occurs. It is, however, conceivable (though improbable) that in spite of the reduction in total respiratory energy released, some may still be available for the low energy needs of active absorption.

In order to test this possibility, the effect of a respiration inhibitor on auxin-induced uptake was investigated. VAN OVERBEEK (**6**) was able to prevent the active absorption of water by tomato roots simply by adding 10^{-4} or 10^{-3} M KCN to the medium. The attempt was therefore made to prevent in this way the auxin-induced water uptake by the potato slices.

TABLE VI

EFFECT OF KCN ON AUXIN-INDUCED WATER UPTAKE. ALL ASEPTIC EXCEPT THOSE MARKED*

CONC.-KCN	AERATION TIME	ORIGINAL FRESH WT. IN GRAMS		FINAL FRESH WT. IN GRAMS		INCREASE (% OF ORIG. FRESH WT.)	
mols.	*hours*	*water*	*IAA*	*water*	*IAA*	*water*	*IAA*
10^{-4}	72	13.955	15.862	21.178	26.883	51.8	56.9
		14.622	15.021	22.367	23.512	52.9	56.5
10^{-3}	72	17.504	20.327	26.940	32.365	53.9	59.5
		16.652	16.648	25.966	26.210	50.0	57.4
10^{-3}	48	16.466	16.372	24.687	25.338	50.0*	54.7*
		16.986	19.571	25.144	31.341	48.1*	60.0*
10^{-3}	72	27.695	26.681	39.527	39.515	42.7	48.1
		23.117	25.935	33.570	37.273	45.2	49.2

Table VI shows that adding KCN to the indole acetic acid failed to prevent the auxin-induced uptake. In the first two sets of results, the KCN was added to the indole acetic acid solution before autoclaving; in the last two, the KCN was sterilized in a hot air oven, then added to the sterilized and cooled indole acetic acid, in order to prevent any possible loss of cyanide. These results again indicate that auxin-induced water uptake is not dependent on respiration.

There is one remaining piece of evidence that can be interpreted as indicating that auxin-induced water uptake is due to active absorption of water. REINDERS (**3, 4**) showed that auxin increases water uptake only under aerobic conditions. When there was no aeration or when nitrogen was bubbled through it instead of air, auxin failed to induce any increased water uptake. Even the controls (in distilled water) did not absorb as much water as when air was bubbled through the medium. REINDERS (**4**) points out that this was sometimes due to irreversible injury causing loss of turgor. In some cases, however, if air was bubbled through after about 2–4 days under anaerobic conditions, the potato discs recovered their original ability to absorb water. It remains to be proved whether this lack of auxin-induced water uptake under anaerobic conditions is directly related to the reduced respiratory energy, or whether it is simply an indirect consequence of a loss of turgidity due to injury suffered under anaerobic conditions. This injury could conceivably be due either to the products of anaerobiosis of the potato cells or to anaerobic organisms growing in the medium.

In our investigations, either no aeration was used, or nitrogen was bubbled through the medium. In either case, after 48–72 hours at 20–23° C., all, or practically all, of the slices were flaccid. Obviously, the lack of auxin-induced uptake could then be at least as logically ascribed to this loss of turgidity (regardless of whether or not it is reversible) as to a reduction of available respiratory energy. Consequently the lack of auxin-induced water uptake under anaerobic conditions has no bearing on the problem of whether or not active absorption is involved.

The purpose of this investigation was to find out whether or not auxin-induced uptake by potato tuber tissues is due to active absorption of water. All the evidence obtained indicates that it is not. Thus the first mode of active absorption (in which an elastic stretch of the cell wall is maintained) is impossible because:

(1) Potato tissues stretch well beyond the elastic limit of their cell walls, even in distilled water.

(2) A steady state is not reached, but instead water continues to be absorbed indefinitely.

(3) Energy is not continuously expended at a greater rate than in the control as would be necessary to retain actively absorbed water.

(4) A marked reduction in the release of respiratory energy induced by a decrease in temperature of 20–25° C. does not result in a loss of water but on the contrary permits a further increased uptake.

(5) The presence of a respiration inhibitor (KCN) fails to prevent the auxin-induced uptake.

Against the second mode of active absorption (in which an initial elastic stretch of the cell wall is followed by permanent set, i.e., is converted into a plastic stretch) is the following evidence: (1) Though the quantity of energy needed to permit such an active absorption is too small to be measured if we assume 100% efficiency, nevertheless there is actually a very marked *reduction* in respiratory energy released accompanying the auxin-induced uptake. (2) When the temperature was dropped to 0–2° C., in most cases the auxin-treated seemed to continue absorbing more water than the controls. (3) The presence of a respiratory inhibitor (KCN) failed to prevent auxin-induced uptake. These results do not, however, yield any information about possible effects of auxin on active absorption of solutes, as Commoner *et al.*'s results seem to point to. They do, however, agree with the results of other investigators in showing that an auxin-induced water uptake can occur in the complete absence of any active solute

TABLE VII

FREEZING POINT LOWERINGS (△) OF POTATO SLICES AERATED IN WATER AND INDOLE ACETIC ACID (10^{-5}) RESPECTIVELY. SOLUTIONS OBTAINED BY EXTRACTING DRIED AND GROUND DISCS WITH ORIGINAL % OF WATER

DATE	△ CONTROL	△ WATER AERATED	△ IAA AERATED
	°C.	°C.	°C.
Sept. 9	0.640	0.620	0.600
Sept. 12	0.755	0.710	0.705
Sept. 17	0.790	0.700	0.690

absorption except possibly of the auxin itself since it is the only solute present in the medium.

Though it is not the purpose of this paper to determine just what is the cause of auxin-induced water uptake by potato tissues, it might be of value to consider the possibilities. Since active water absorption is apparently not involved, this leaves three possible explanations: increased osmotic pressure, decreased wall pressure, and increased protoplasmic hydration. REINDERS (**4**) favors the first of these but it has since been disproved by VAN OVERBEEK (**7**). Results obtained in this investigation are in agreement with van Overbeek (table VII). The possibility of increased protein hydration can be evaluated from the following calculations. Only 2% of the dry matter of the potato discs is protoplasmic protein (**1**), or 0.4% of the fresh weight of the cell. And since about 10% of protoplasm consists of proteins, the protoplasm is about 4% of the fresh weight of the cell. If all the auxin-induced uptake were due to increased protoplasmic hydration, a 5% uptake (which is readily obtained) would involve more than doubling the original hydration of the protoplasm, over and above the approximate doubling that occurs in distilled water. This seems hardly

likely. At any rate such a large increase in hydration capacity should be easily detected experimentally.

The freeze-drying used in the above experiments does not remove all the water. To determine how much moisture was still held, the slices were subsequently dried at 110° C. If protoplasmic hydration is the cause of the auxin-induced uptake, one might expect a greater hydration in the freeze-dried condition. Results fail to reveal such a difference (table VIII).

One question should be clarified before attempting to explain these results,—was the water uptake accompanied by cell division or only by cell elongation? REINDERS (**4**) failed to find cell division until the discs had been aerated for four days, and even then it was slight. Nearly all of the

TABLE VIII

WATER (% OF OVEN-DRY WT.) RETAINED BY POTATO SLICES AERATED IN WATER AND INDOLE ACETIC ACID (10^{-5}) RESPECTIVELY AND SUBSEQUENTLY DRIED IN VACUO OVER $Mg(ClO_4)_2$.

DATE	AERATED IN WATER	AERATED IN IAA
Apr. 28	4.5	4.3
May 3	4.35	4.7
May 9	2.95	2.3
May 14	4.2	4.45
May 20	3.85	3.5
May 24	2.5	3.15
June 27	5.2	3.4
June 27	4.15	5.6
July 4	4.6	3.2
Average	3.99	3.84

tests made in this investigation were for shorter periods. Sections made at the end of aeration failed to reveal any but a trace of cell division.

By a process of elimination, it would appear that only decreased wall pressure (i.e., increased wall plasticity) can explain the auxin-induced water uptake. Furthermore, the very large variation in this quantity is understandable on the basis of a discontinuous, jerky, and irreversible plastic stretch of the cell wall, but difficult to explain in terms of a continuous increase in active water absorption or protoplasmic hydration. The fact that equilibrium has never been attained (the increased uptake continuing apparently indefinitely) is explainable only on the basis of cell wall plasticity.

Summary

1. Theoretically there are two possible modes of active water absorption due to auxin: one involving an elastic stretch of the cell wall, and the other an initial elastic stretch that is then converted into a permanent set (i.e., a plastic stretch).

2. Calculations reveal that the second mode of active water absorption

would require a negligible expenditure of energy, assuming 100% efficiency.

3. Auxin-induced water uptake at room temperature is not lost by subsequent transfer to 0–2° C. for 24 hours, but may actually increase.

4. Respiratory loss of dry matter is markedly reduced by auxin at 21–24° C. though water uptake is increased. At 25–29° C. respiratory loss is increased by auxin.

5. 10^{-4} M and 10^{-3} M KCN has no effect on auxin-induced water uptake.

6. The osmotic pressure at the end of the experiment is the same for slices aerated in IAA as for slices aerated in distilled water, provided only the original water content is present.

7. Calculations reveal that the original protoplasmic hydration would have to be increased by over 100% in order to account for the auxin-induced uptake. Yet there is no difference between the amount of water held by slices dried at room temperature after aeration in IAA and water respectively.

8. All these results indicate that auxin-induced water uptake is not due to active absorption, to increased osmotic pressure, or to increased protoplasmic hydration.

BOTANY DEPARTMENT
UNIVERSITY OF MISSOURI
COLUMBIA, MISSOURI

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THE EFFECT OF CALCIUM AND OTHER IONS ON THE EARLY DEVELOPMENT OF THE RADICLE OF COTTON SEEDLINGS[1]

J. T. Presley[2] and O. A. Leonard[3]

(WITH THREE FIGURES)

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Introduction

Considerable difficulty has been experienced in growing cotton seedlings with healthy radicles for laboratory experiments. The usual method of growing the seedlings was to germinate disinfected acid delinted cotton seed between sheets of moist filter paper in Petri dishes or moist chambers. The filter paper was moistened and the excess water poured off; then the cotton seeds were placed in the dishes. The seedlings were removed from the dishes when the radicles were one-half to one inch long and transferred to containers filled with tap water. Paraffined cheesecloth was used to cover the containers and the radicles were placed in the tap water through holes in the cover. After a few days the seedlings were developed sufficiently to be transferred to containers of nutrient solutions. Usually a high percentage of the seedlings were not usable because of unhealthy radicles. The unhealthy condition of the radicles was at first considered the result of bacterial breakdown, but when every precaution was taken to maintain aseptic conditions the breakdown still occurred. It was then thought that perhaps unfavorable temperatures or insufficient aeration might encourage the breakdown, but preliminary tests indicated that neither temperature nor aeration was entirely responsible for the unhealthy condition of the radicles. It was found, however, that the radicles of seedlings germinated under relatively high moisture tension were extremely susceptible to breakdown. It was also found that the concentration and kind of ions added to the solution into which the radicles were placed had a pronounced effect upon the health and development of the radicles. This paper describes the various experiments made.

Procedure

Cotton seeds were germinated at high and low moisture levels and the seedlings transferred to tap water or distilled water containing various salts singly and in combination. Acid delinted cotton seeds of the Half and Half variety were surface sterilized with a solution of sodium hypochlorite (57 gm. per l.) for two minutes. The excess sodium hypochlorite was removed

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[2] Pathologist, Division of Cotton and Other Fiber Crops and Diseases, BPIS&AE, U. S. Dept. of Agriculture, cooperating with the Mississippi Agricultural Experiment Station.

[3] Plant Physiologist, Mississippi Agricultural Experiment Station.

with tap water and the seeds were placed in sterile Petri dishes, 12 seeds per dish. The Petri dishes contained three sheets of no. 2 filter paper, one sheet in the top of the dish and two in the bottom. In all of the experiments the top filter paper was moistened with 1.5 cc. of sterile water and 3 cc. or 6 cc. added to the bottom filter papers. (In a germination test it had been found that 3 cc. of water per Petri dish was adequate for germination but the seedlings appeared to be under moisture stress. For the purpose of the experiments, the 3 cc. level will be considered low moisture and the 6 cc. level will be considered high moisture.) The Petri dishes (4–6 for each treatment) were transferred to an incubator where the temperature was maintained at 26–28° C. After two or three days the seedlings were removed from the Petri dishes and placed in water through paraffined cheesecloth. The plants were observed for varying periods up to several weeks, but primary interest was in the results after about five days.

The tap water used in these experiments had the following composition in parts per million: SiO_2, 15; Fe, 0.3; Ca, 8.2; Mg, 1.6; Na, 39; CO_3, 3.6; HCO_3, 106; SO_4, 1.6; and Cl, 18.

The radicles of the seedlings germinated at the low moisture level appeared to be healthy when transferred to tap water; but they were appreciably smaller than those germinated at the high moisture level, and absorbed water to the extent that air was driven from the intercellular spaces and the radicles became translucent, or watersoaked, in appearance. No such change was noted in the radicles of seedlings germinated at the high moisture level. In the seedlings from the low moisture treatment there was immediate injury and subsequent breakdown of most of the radicles. In tap water with calcium added the radicles appeared to remain more healthy than those in tap water alone. Those in tap water with magnesium added were completely broken down at the end of the experimental period. The seedlings in tap water with both calcium and magnesium added appeared to be slightly inferior to those in tap water alone. Most of the seedlings germinated at the high moisture level, when placed in tap water alone, or in tap water with calcium added, had rapidly growing healthy radicles, whereas those in tap water with magnesium added were distinctly unhealthy. The seedlings in tap water with both magnesium and calcium added appeared to be as healthy as those in tap water alone.

COMPARISON OF MOISTURE LEVEL AND VARIETY OF COTTON ON BREAKDOWN OF RADICLES

Varieties	Treatments	Molar concentration	Moisture level	
Half and Half, Coker 100 Wilt, Hartsville, Sea Island, American Egyptian	Tap H_2O		3 cc.	6 cc.
"	Tap H_2O + $MgSO_4$	0.004 M	"	"
"	Tap H_2O + $CaCl_2$	0.002 M	"	"

Three varieties of upland cotton (*Gossypium hirsutum*) were used and two of the barbadense type (*G. barbadense*). The rate of germination of

the upland cottons was slightly reduced by high moisture, the effect on Hartsville being most pronounced. On the other hand, Sea Island and American Egyptian germinated just as well and as rapidly at the high as at the low moisture level. The radicles of all the varieties germinated at low moisture absorbed water so rapidly that the air in the intercellular spaces was driven out as in experiment 1. After five days the condition of the radicles of the various varieties and at the different moisture levels was noted. Most of the radicles of seedlings of the upland varieties that germinated at the high moisture level were in a vigorous growing condition, while those germinated at low moisture were unhealthy. The Sea Island and American Egyptian varieties seemed to be somewhat resistant to breakdown at the low moisture level.

INFLUENCE OF TEMPERATURE ON BREAKDOWN OF RADICLES

Treatments	Molar concentration	Moisture level		Temperature		
Tap H_2O		3 cc.	6 cc.	at 10° C.	27° C.	30° C.
Tap H_2O + $MgSO_4$	0.004 M	"	"	"	"	"
Tap H_2O + $CaCl_2$	0.002 M	"	"	"	"	"

In order to study the effect of temperature as related to breakdown, a series of cotton seedlings was germinated at high and low moisture levels, also with added calcium and magnesium. These seedlings were transferred as in previous experiments to containers of tap water and tap water plus calcium and magnesium. In this experiment the solutions were held at 10°, 27°, and 30° C. for two days after the seedlings were transferred to the various solutions and then were held at room temperature (27° C.) for an additional three days. The breakdown of the radicles when germinated at low moisture was not affected by temperature. It was thought that reducing the temperature might reduce the rate of absorption of water by the radicles as well as the hydrostatic pressure that might develop. Apparently the effect of temperature on water entry and hydrostatic pressure or tissue breakdown was negligible. The influence of calcium and magnesium in this experiment did not differ from the previous experiments. Radicles from seedlings germinated at high moisture were uniformly healthy except for the apparent toxicity of magnesium. The length of the radicles was very markedly affected by temperature. The greatest elongation occurred at 30° and the least at 10°.

Distilled water was used in this experiment in order that the effect of anion as well as cation on the development of the radicles could be studied. This experiment showed a striking toxicity of magnesium salts when used alone, and an equally striking benefit of calcium salts whether used alone or in combination with different molarities of magnesium salts. The anions, chloride and sulphate, had no pronounced effect on the development of the radicles. The data further showed that distilled water was a poor medium

EFFECT OF ANION, CATION, AND MOISTURE ON BREAKDOWN OF RADICLES

Treatments	Molar concentration			Moisture level	
				3 cc.	6 cc.
Dist. H_2O					
Tap H_2O				"	"
Dist. H_2O + $MgSO_4$	.002 M	.004 M	.008 M	"	"
Dist. H_2O + $CaSO_4$	"	"	"	"	"
Dist. H_2O + $MgCl_2$	"	"	"	"	"
Dist. H_2O + $CaCl_2$	"	"	"	"	"
Dist. H_2O + $MgSO_4$ + $CaCl_2$	"			"	"

for the early development of cotton radicles, probably because of calcium deficiency. Tap water which contains 8.2 p.p.m. calcium was superior to distilled water.

EFFECT OF MONOVALENT AND DIVALENT CATIONS AND MOISTURE ON BREAKDOWN OF RADICLES

Treatments	Concentrations			Moisture level	
				3 cc.	6 cc.
Dist. H_2O					
Dist. H_2O + $CaSO_4$	0.001 M	0.016 M		"	"
Dist. H_2O + $MgSO_4$	"	"		"	"
Dist. H_2O + K_2SO_4	0.002 N	0.008 N		"	"
Dist. H_2O + Na_2SO_4	"	"		"	"
Dist. H_2O + $CaSO_4$	0.001 M	+ $MgSO_4$	0.004 M	"	"
Dist. H_2O + $CaSO_4$	"	+ "	0.008 M	"	"
Dist. H_2O + $CaSO_4$	0.002 M	+ "	0.002 M	"	"
Dist. H_2O + $CaSO_4$	"	+ K_2SO_4	0.002 N	"	"
Dist. H_2O + $CaSO_4$	"	+ Na_2SO_4	"	"	"
Dist. H_2O + K_2SO_4	0.002 N	+ "	"	"	"

In previous experiments with the divalent ions of calcium and magnesium, it was demonstrated satisfactorily that calcium deficiency and not ion toxicity was related to the early breakdown of the cotton radicles. This test was designed to further explore the possibility that antagonistic effects might be shown by monovalent cations or certain combinations of monovalent and divalent cations. The results of the experiment are shown by photographs of the seedlings in figures 1, 2, and 3. The results indicate again that the addition of cations to the distilled water without the addition of calcium produced only unhealthy radicles. Calcium added to solutions containing magnesium, sodium, or potassium reduced or inhibited the toxic

EFFECT OF MOISTURE LEVEL ON THE RADICLES OF COTTON SEEDLINGS GROWN IN SOIL AND SAND

Treatments	Per cent. moisture			
Coarse sand	3	6	9	12
Fine sand	"	"	"	"
Ruston sandy loam, top soil	"	"	"	"
Ruston sandy loam, 2 ft. depth	"	"	"	"
Ruston sandy loam, 4 ft. depth	"	"	"	"
Ruston sandy loam, 6 ft. depth	"	"	"	"

effect of the other ions. The ratio of calcium to magnesium did not appear to be of immediate importance. However, after the seedlings had grown for a week in a solution high in magnesium (.008M) and low in calcium

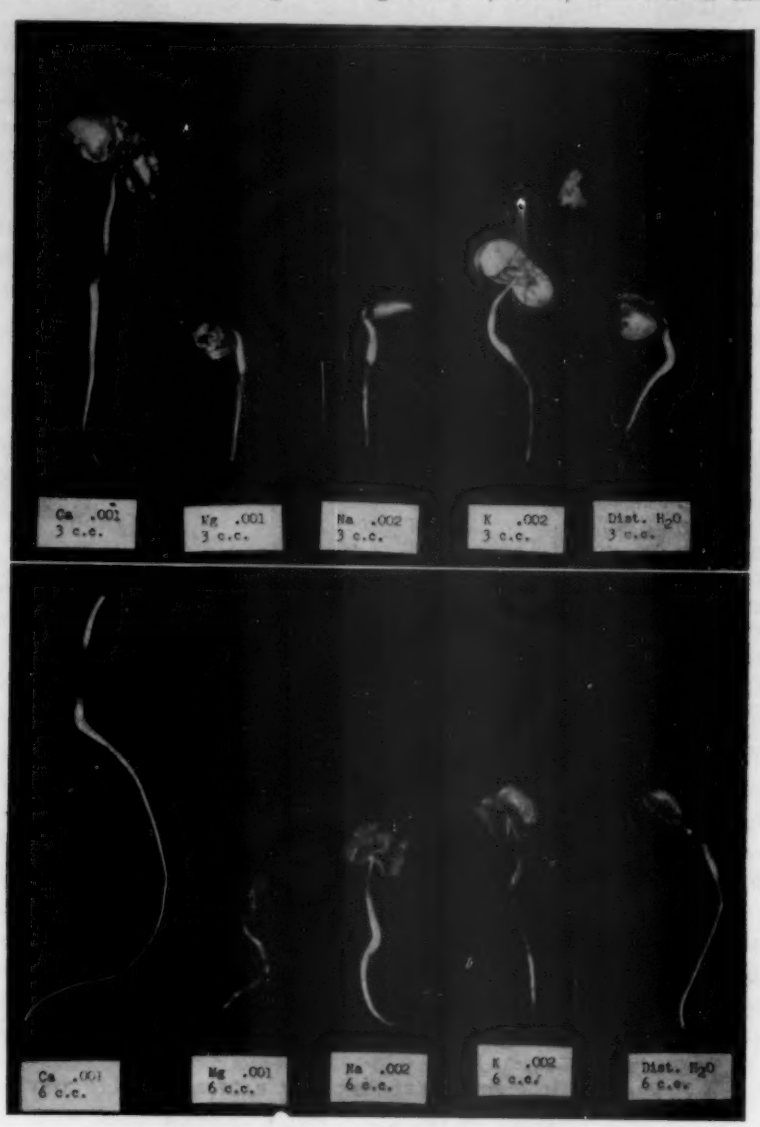

FIG. 1. Cotton seedlings germinated at low and high moisture levels (3 cc. and 6 cc.) five days after being placed in distilled water and single salt solutions containing monovalent and divalent cations. The divalent cations, Ca and Mg, are expressed in molar concentrations; the monovalent cations, Na and K, are expressed in normal concentrations. Note the beneficial effect of calcium.

(.001M), there appeared to be some magnesium injury. Radicles of seedlings germinated at low moisture levels were less affected by water-soaking when placed in solutions containing calcium.

This preliminary experiment was conducted to determine the influence of various moisture levels and subsequent high moisture level after germination on the radicles of cotton in soil and sand. After emergence of the

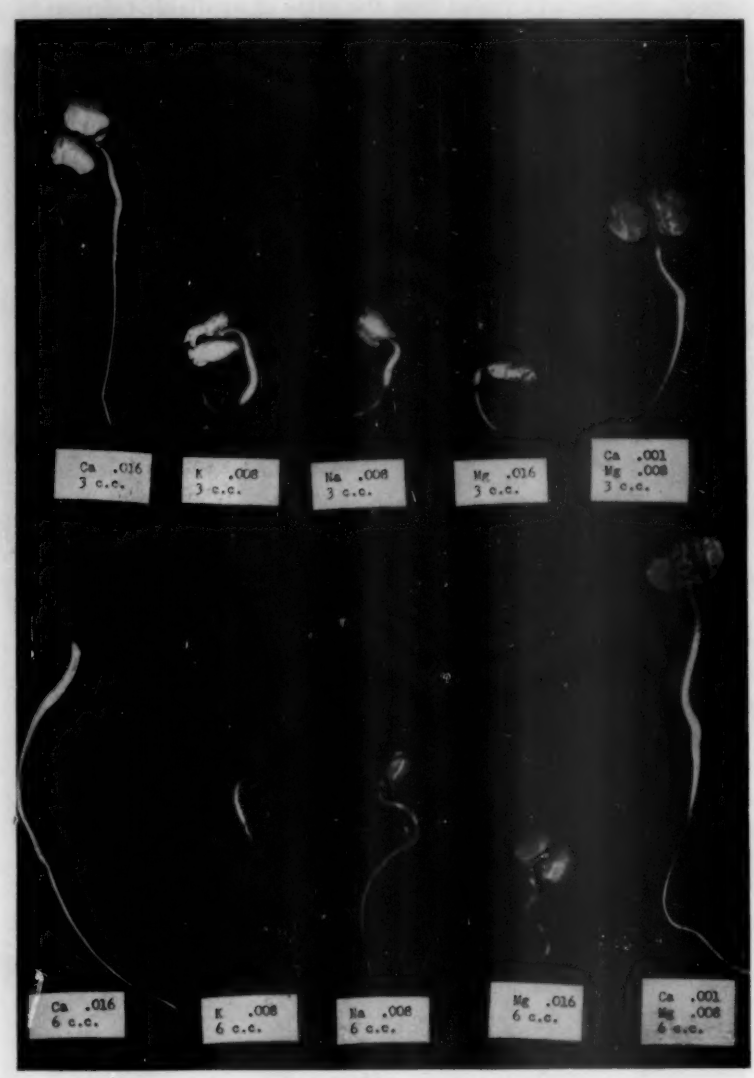

FIG. 2. Cotton seedlings germinated at low and high moisture levels (3 cc. and 6 cc.) five days after being placed in solutions containing monovalent and divalent cations. Concentrations expressed as in figure 1.

seedlings the moisture in all treatments was raised to 30 per cent. The results seem to be comparable with the water culture experiments in respect to moisture level and calcium level. In soil or sand that was high in calcium there was little permanent effect on the radicles at the low moisture level.

Soil that was low in calcium and relatively high in magnesium (Ruston sandy loam subsoil) produced very poor radicles, particularly at the low moisture level. In this connection it is interesting to note that TIEDJENS and SCHERMERHORN (7) found that the ratio of available calcium to potas-

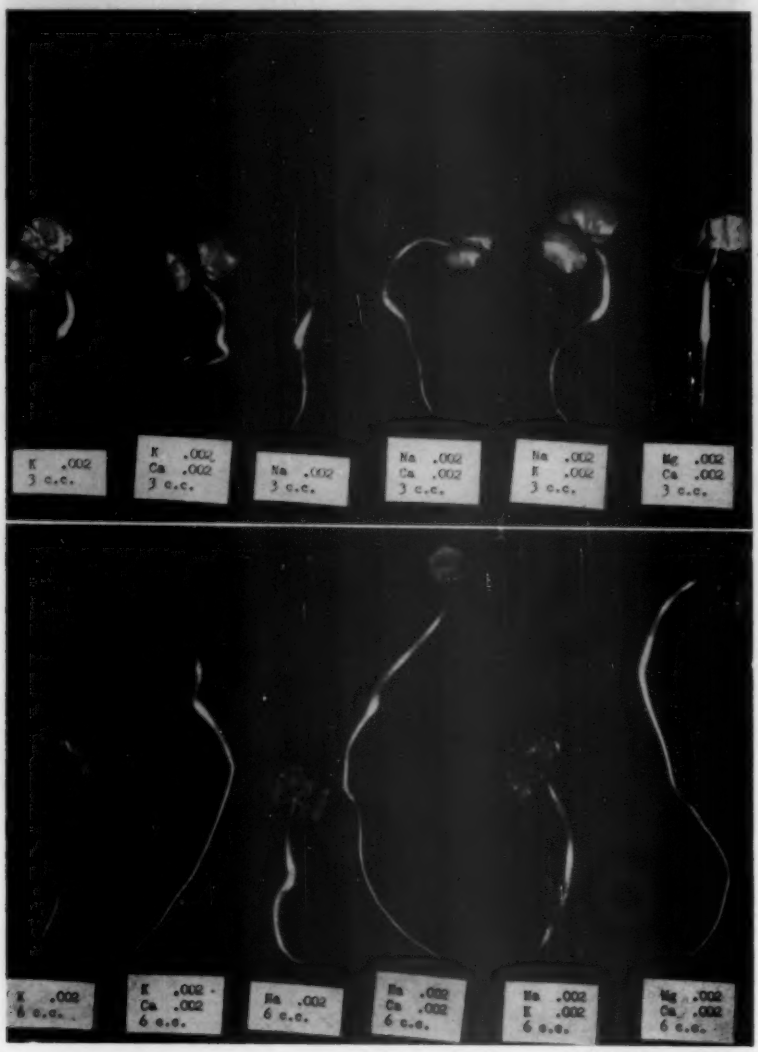

FIG. 3. Cotton seedlings germinated at low and high moisture levels (3 cc. and 6 cc.) five days after being placed in solutions containing monovalent and divalent cations. Concentrations expressed as in figure 1.

sium and sodium was extremely important for the germination of seed and growth of vegetable crops on coastal plain soils. ALBRECHT (1) demonstrated a beneficial effect of calcium on tomato seed germination in soil either in the presence or absence of commercial fertilizer.

Discussion

Cotton seedlings that are grown at a low moisture level produce radicles that absorb water excessively, and air is driven from the intercellular spaces. As the radicles absorb water and expand, they become translucent or water-soaked in appearance. The water-soaked tissues, apparently, are very susceptible to fungal or bacterial breakdown. When seedlings germinated at low moisture level are placed in tap water or distilled water containing calcium salts, a high percentage of the radicles remain healthy. However, if they are placed in tap water or distilled water containing magnesium, potassium, or sodium salts, the radicles break down more rapidly than when they are placed in tap water or distilled water alone. SEIFRIZ (**5**) indicated the protective influence of calcium on torn protoplasm by stating, "There is further experimental evidence that a torn protoplasmic surface is repaired only in the presence of calcium owing to the aggregating (gelating) effect of the latter, an effect that sodium lacks." The small amount of calcium in tap water apparently is not sufficient to prevent breakdown of radicles grown at a low moisture level.

The swelling of the radicles after being placed in water is undoubtedly the result of high osmotic pressure developing within the root cells of seedlings grown under conditions of moisture stress. The radicles of seedlings grown under conditions of moisture stress contained approximately 2½ times as much alcohol soluble material as did those grown under conditions of low moisture stress.

Cotton seedlings that are germinated at a low moisture stress produce radicles of greater diameter than those that develop under high moisture stress. Upon being placed in tap water, the radicles developed at a low moisture stress absorb water, but not to the extent of displacing all of the air from the intercellular spaces. When a calcium salt is added to the tap water all of the radicles remain healthy and grow rapidly, even more rapidly than in tap water alone. In this connection it is interesting to note the experience of SOROKIN and SOMMER (**6**) with the common pea, *Pisum sativum* var. golden vine, "Since the effect of the absence of calcium showed so rapidly and the plants were so badly injured, it was found desirable to germinate the seeds and keep them in a solution of calcium sulphate until the primary root reached the length of 1½ inches."

In order to study the effect of different ions upon cotton seedling radicles it was necessary to use distilled water. These studies showed that calcium is indispensable for healthy radicles. In distilled water the radicles became unhealthy and gradually broke down completely. Sodium, potassium, and magnesium, when used without calcium, greatly accelerated the rate of breakdown over that in distilled water alone. Magnesium was particularly detrimental. The injurious effect of magnesium is well known on radicles in single salt solutions and toxicity is out of proportion to the influence on osmotic pressure. This was strikingly shown by WADLEIGH and GAUCH (**10**), working with guayule. LOEB's (**4**) explanation of a physiologically

balanced solution was that cations enter into combination with protoplasm and can be displaced by other kinds of cations from external solutions in accordance with the law of mass action. The explanation by Loeb may partly explain the pattern of behavior of cotton radicles in the present experiments. GAUCH (**3**) observed that bean seedlings made little or no growth in any solution unless calcium was added. Magnesium salts alone were very injurious, apparently because in plant tissues calcium is not as readily translocated as other minerals. Seedlings can suffer from calcium deficiency quickly [BAMFORD (**2**)]. TRUE and BARTLETT (**9**) and TRUE (**8**) demonstrated the importance of calcium salts which permitted seedlings of *Lupinus albus* to retain possession of salts already present in the tissues.

Evidently the cotton radicles are sufficiently deficient in calcium so that a single salt solution of calcium produces no detrimental effect within the experimental period. Given sufficient time, calcium alone should be detrimental to a seedling, based on the law of mass action. Magnesium and potassium would ultimately be displaced in the protoplasm resulting from the relative deficiency of one or more of these cations.

Although the effect of high moisture stress during germination and the effect of calcium and magnesium salts vary somewhat with different varieties of cotton, the over-all trends are similar.

Seedlings of oats (*Avena sativa*), corn (*Zea mays*), peas (*Pisum sativum*), lima beans (*Phaseolus lunatus*), vetch (*Vicia faba*), soybeans (*Soja max*), peanuts (*Arachis hypogaea*), and cotton (*Gossypium hirsutum*) were germinated under satisfactory moisture conditions and placed in single salt solutions of calcium sulphate, magnesium sulphate, and distilled water. The detrimental effect of magnesium occurred in all cases but the radicles of upland cotton broke down more rapidly than the radicles of other seedlings. No ill effect of distilled water was noted for any of the seedlings except cotton. The radicles of cotton became greenish in color and were not vigorous. All of the various types of seedlings in the calcium sulphate solution appeared vigorous and were, in general, superior to those in distilled water.

The above results suggest that the young radicles of upland cotton are particularly sensitive to calcium deficiency and may be useful plants to employ for studying calcium deficiency or ion toxicity.

Summary

1. Seeds of upland cotton of the Half and Half variety were germinated in Petri dishes at high and low moisture levels. Cations were varied in the water used in germinating the cotton seeds as well as in the solutions in which the seedlings were subsequently placed.

2. The radicles of seeds germinated under low moisture conditions absorbed an excess of water when they were transferred to the various solutions. The air in the intercellular spaces was displaced. After four days the radicles were mostly broken down by secondary bacterial action.

3. Calcium salts added to tap water or distilled water resulted in a higher percentage of healthy radicles.

4. Radicles developing at the high moisture levels did not absorb water to the extent of crowding out all of the air from the intercellular spaces. When adequate calcium was present these radicles remained healthy and grew rapidly.

5. In distilled water the radicles became unhealthy, and some breakdown was apparent in about four days. The progress of the breakdown was appreciably hastened by salts of magnesium, sodium, or potassium.

6. The upland varieties of cotton tested appeared to be more sensitive to calcium deficiency than any of the other seedlings tested.

7. The two conditions necessary for producing cotton seedlings with healthy radicles are adequate moisture during the germination period and adequate calcium during early stages of growth.

MISSISSIPPI AGRICULTURAL EXPERIMENT STATION
STATE COLLEGE, MISSISSIPPI

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THE IDENTIFICATION OF ETHYLENE AS A VOLATILE PRODUCT OF RIPENING AVOCADOS[1]

HARLAN K. PRATT, ROY E. YOUNG, AND J. B. BIALE

(WITH ONE FIGURE)

Received January 12, 1948

Introduction

The production of a physiologically active emanation by ripening fruits has frequently been demonstrated by biological tests, including leaf epinasty, respiration effects, potato sprout abnormalities, and the triple response of etiolated legume seedlings (**11**). HUELIN (**8**) first noted the analogy between these effects and those produced by small concentrations of ethylene, and GANE (**4, 5**) first showed by chemical means that the active volatile substance produced by apples was actually ethylene gas. An inferential proof, partly chemical and partly biological, that ethylene is produced by pears was achieved by HANSEN and HARTMAN (**7**), and NIEDERL, BRENNER, and KELLEY (**10**) demonstrated ethylene production by bananas. PRATT and BIALE (**11**) found that the climacteric rise in carbon dioxide production by ripening avocado fruits was accompanied by a cycle of production of a gas, presumably ethylene, which caused the triple response of etiolated pea seedlings. The purpose of the present study was to identify chemically this active emanation and to develop a technique which would simplify the isolation and identification of ethylene as a probable volatile product of other fruits.

Apparatus for absorbing ethylene

GAS SCRUBBING APPARATUS

The most effective reagent for the removal of minute quantities of ethylene from a gas stream is bromine, which is difficult to manage being decidedly volatile and noxious. The apparatus of NIEDERL, BRENNER, and KELLEY (**10**) was tried, but the charge of bromine was lost from the apparatus within a few hours, even with temperatures lower than they specified. This problem was solved as follows[2] (Fig. 1): The efficient spiral absorber (H) of MARTIN and GREEN (**9**) was charged with liquid bromine (30% by volume) dissolved in a solvent (methylene chloride), and above the absorber was a "cold finger" trap (J) chilled with pulverized dry ice in acetone supplied from a large vacuum-jacketed reservoir (K). As the moving gas stream evaporated the bromine, it was carried to this trap on which it con-

[1] A cooperative project of the Division of Subtropical Horticulture and the Division of Truck Crops, College of Agriculture, University of California, carried out in the laboratories of the former division.

[2] The operating principle suggested by Dr. John D. Roberts, Massachusetts Institute of Technology.

densed by freezing. The volatile solvent, also carried to this surface, was condensed to liquid and redissolved the bromine, washing it back into the absorber. This apparatus permitted continuous operation with no loss of absorbent solution and no bromine odor nuisance in the laboratory. The

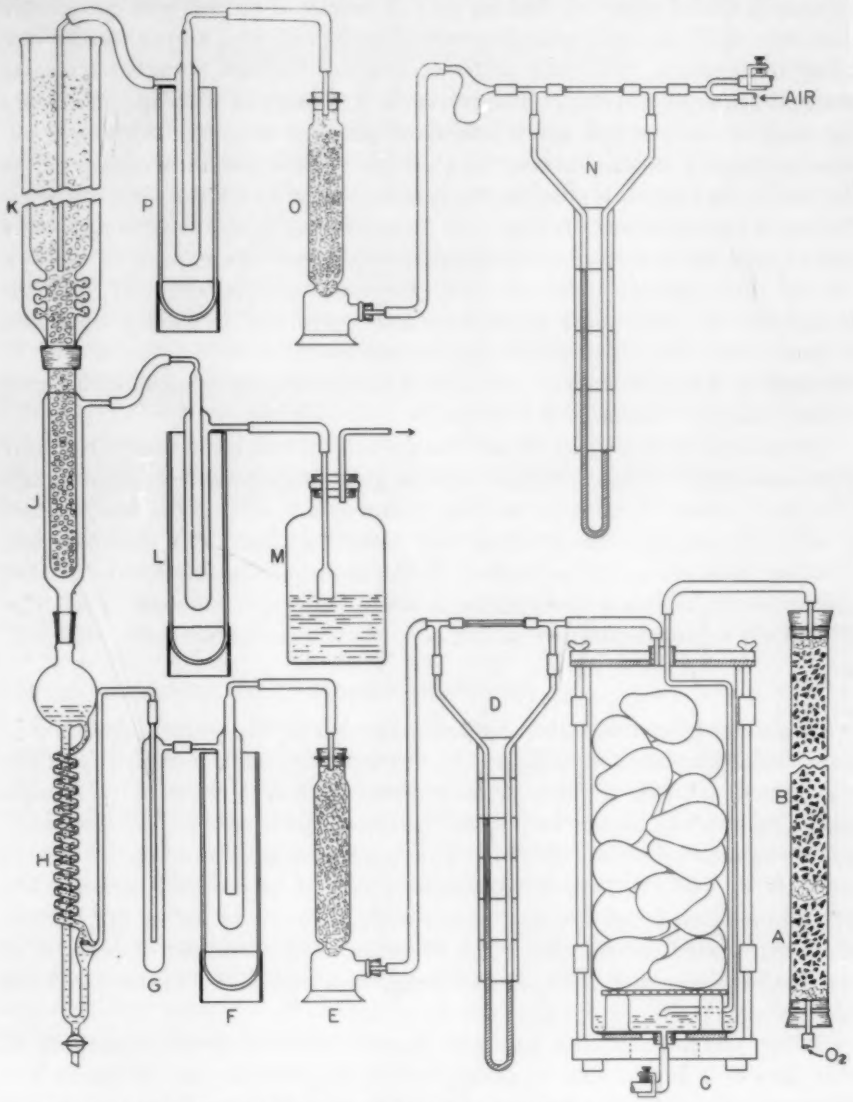


FIG. 1. Absorption train for the collection of ethylene from avocados.

efficiency of the absorption apparatus was verified with an ethylene concentration of 1000 p.p.m., by passing the scrubbed gas stream over etiolated pea seedlings; no response was obtained, showing removal of ethylene to less than one-tenth part per million.

Important dimensions of the absorption apparatus were as follows: The spiral absorber consisted of twenty-seven turns of six millimeter tubing and had a capacity of 100 ml. of solution. The "cold finger" of the trap was 24 cm. long with a diameter of 4 cm. enclosed in a six cm. outer jacket. The vacuum-jacketed reservoir had an overall height of 80 cm. with an outside diameter of 10 cm., an inside diameter of eight cm., and a lower outlet diameter of four cm., the lower outlet connecting through a rubber sleeve to the cold finger of the trap. The reservoir was charged with approximately one liter of acetone and seven pounds of crushed dry ice. Although acetone is superior to ethyl alcohol as a vehicle since it prevents caking of the dry ice in the reservoir, stirring was required to move a steady supply of refrigerant into the trap. To this end, three liters of dry air per minute were passed into the reservoir at the constriction above the expansion bellows, via the flowmeter (N), the calcium chloride drying tower (O), and the freezing trap (P). This large reservoir permitted overnight operation without attention. The entire apparatus was kept in a room at 15° C. throughout the experiment. All critical connections were made with Pyrex tubing held end to end with "Tygon."

The choice of methylene chloride as the solvent was important. Not only must the solvent be inert, but its physical properties must be in appropriate relation to those of bromine at room temperature and at the temperature of dry ice to secure the washing and return action. The inertness and freedom from olefin contamination of the absorbent solution were verified before use by allowing the mixture to stand two days, followed by distillation at 40° C. and a pressure of 350 mm. of mercury—no residue remained.

Absorption train

The absorption train used for the collection of the active emanation of avocados is diagrammed in figure 1. Oxygen from high pressure cylinders was passed through a tower of brominated activated charcoal (Columbia 4ACW, size 6/14) to remove possible active contaminants by the method of Southwick and Smock (**12, 13**). This tower consisted of a tube 50 mm. in diameter and 80 cm. long filled one-third full of brominated carbon (A): (24 ml. of bromine on 200 gm. of carbon), followed by twice this volume of unbrominated carbon (B). The efficiency of this method of removal of possible contamination from the gas stream was verified by the etiolated pea test.

After purification, the gas was passed over the fruit, contained in nine jars (C) (connected in series) of the type described by Biale and Shepherd (**2**). In the bottom of each jar, except the first, was placed 500 ml. of 6-normal sodium hydroxide to absorb the carbon dioxide of respiration, and this was replenished as needed via the drain in the bottom of each jar. The net flow of gas through the absorber, after the appreciable absorption of oxygen by the fruit, was held at 350 ml. per minute through the flowmeter (D). After passing the fruit, the gas stream was dried by a tower of anhydrous calcium chloride (E) and a trap in dry ice-acetone

(F) and then entered the gas scrubbing apparatus described above, consisting of a safety trap (G), a spiral absorber (H), and a "cold finger" dry ice trap (J). Following the absorber was another dry ice-acetone trap (L) and a trap (M) containing sodium hydroxide to prevent any possible escape of bromine vapors.

Experimental procedure and results

HANDLING OF THE FRUIT.—During February, 1947, mature preclimacteric Fuerte avocados were harvested from the experimental orchard of the College of Agriculture, Los Angeles, and twenty-five were placed in each of nine respiration jars (figure 1, C), giving a total fruit weight of 57.0 kg. The fruit was kept at 15° C. throughout the experiment. Since the rate of respiration of avocados is very high, a stream of air at a rate low enough to permit efficient absorption could not provide enough oxygen for the normal metabolism of such a large sample. BIALE (1) showed that the behavior of avocados in pure oxygen was not significantly different from that in air. Therefore, a stream of oxygen (instead of air) was passed over the fruit throughout the experiment. All fruits in composite fruit samples usually seem to enter the respiration climacteric simultaneously, presumably due to its stimulation in all fruits by the production of ethylene in the first fruit to ripen. To expedite the experiment, this effect was simulated by subjecting the jars of experimental fruit to the emanations of ripe avocados for twenty hours before starting the run.

Absorption from the experimental lot was started on the third day and continued for a total of 139 hours. Twice during this period the alkali in the jars was replaced. The fully ripe stage (fruit soft and edible) was reached on the fifth day of the run (the eighth day in storage).

RECOVERY OF THE PRODUCT.—At the end of the absorption period, the solvent and most of the excess bromine were removed from the reaction mixture by distillation at 40° C., starting at a pressure of 600 mm. of mercury and gradually reducing to 300 mm. When approximately five ml. of liquid (mostly bromine) remained, the still was dismantled, and the remaining free bromine removed by cautious addition, with cooling, of sodium bisulfite solution. A small amount of yellow oil separated from the bisulfite solution. This mixture was extracted three times with ether which was in turn washed with half-normal hydrochloric acid and dried over anhydrous calcium chloride. The ether was removed by slow aeration, leaving 650 mg. of light yellow oil.

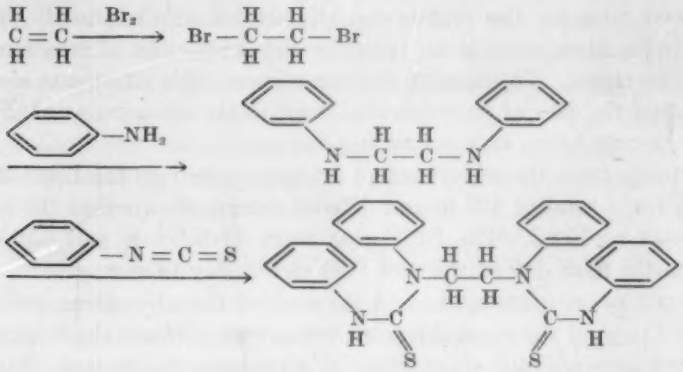
PREPARATION OF DERIVATIVES.—Following the method of GANE (**4, 5**), 6 ml. of redistilled aniline were added to the entire sample of unknown oil, and the mixture heated for four hours on a boiling water bath. The excess aniline was then driven off by steam distillation, leaving a dark red gummy phase which was separated by chloroform extraction. Removal of the chloroform by aeration left a brown crystalline deposit. This was recrystallized from 50% ethyl alcohol, giving white lustrous flakes melting at 64.4–65.3° C. (uncorrected). Recrystallized N,N′-diphenylethylenedia-

mine (Eastman White Label) melted at 64.3–65.3° C., and the known and unknown mixed at 64.2–65.0° C. (uncorrected).

The above derivatives were converted to N,N'-diphenyl-N,N'-dianilinothioformyl ethylenediamine by the procedure of DAVIS (**3**). Approximately 75 mg. of each of the aniline derivatives were mixed in test tubes with 2 ml. of phenylisothiocyanate and heated to boiling for eight minutes. After standing overnight, the reaction mixtures were completely solid. The products were then washed three times with petroleum ether, followed by three washings with 95% ethyl alcohol, and were recrystallized from benzene. Melting point data (uncorrected): From Fuerte avocado derivative, 187.4–188.0° C.; from N,N'-diphenylethylenediamine (Eastman White Label), 187.2–188.0° C.; mixture of these, 187.4–188.0° C.

Discussion

The melting point and mixed melting point data of the derivatives described above show that the expected sequence of reactions has indeed occurred, and that ethylene was therefore present as a volatile product of the ripening fruit:



It is interesting to estimate roughly the quantity produced. If the 650 mg. of oil may be assumed to be pure ethylene dibromide, we have the equivalent of 77.5 ml. of ethylene which was recovered from 2920 liters of gas, an average concentration of 26.5 parts per million. This also represents a total production of 0.34 ml. per fruit of 253 gm. average weight, or 1.4 ml. per kg. This may be contrasted with the estimates of 0.0033 ml. per kg. of bananas (**10**) and with 5 ml. per kg. of apples (**4, 5**). PRATT and BIALE (**11**) showed that the peak of ethylene production coincided with the respiratory peak. This presumably occurred during the third day of absorption, at which time determination of ethylene by the microbromination method of HANSEN (**6**) on a sample of the gas stream from the jars of fruit gave about 135 parts per million. Similar results were obtained in an experiment with Nabal, the leading summer variety of avocados.

Summary

An efficient and convenient method was devised for absorbing and identifying ethylene from low concentrations in gas streams. Using this tech-

nique, ethylene was identified as a volatile product of ripening avocado fruits.

DIVISION OF TRUCK CROPS
UNIVERSITY OF CALIFORNIA
DAVIS, CALIFORNIA
DIVISION OF SUBTROPICAL HORTICULTURE
LOS ANGELES 24, CALIFORNIA

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A COMPARISON OF ELECTRIC RESISTANCE UNITS FOR MAKING A CONTINUOUS MEASUREMENT OF SOIL MOISTURE UNDER FIELD CONDITIONS[1]

G. J. Bouyoucos and A. H. Mick

(WITH FOUR FIGURES)

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Since its introduction in 1940, the plaster of Paris block method of measuring soil moisture by means of electrical resistance (**4**) has been widely employed in agricultural fields. Hydrologic research[2] (**16**), war production problems involving guayule culture (**11, 12**), and many other investigations ranging from field irrigation to greenhouse studies have made use of this technique. Briefly, the method consists of imbedding within the soil a plaster of Paris block containing two electrodes. At a constant temperature, the electrical resistance of this absorbent material varies with its moisture content, which, in turn, varies according to the magnitude of the forces exerted by the surrounding soil. A determination of block resistance thus gives a measure of these soil moisture forces, a measurement which can be made with a high degree of accuracy and reproducibility within the moisture range that is of significant importance to growing plants (**12**).

Fundamental principles

Because an absorption block indicates the force with which moisture is held by the soil environment, it may be considered to portray more accurately than conventional volume or weight percentages soil moisture conditions as they exist with respect to actively transpiring plants. The conventional percentages have, of course, long been recognized as inherently fallacious in this regard because they cannot express free energy factors operating in moisture phenomena. Figure 1 is a typical curve in the soil moisture-block resistance relationship. It illustrates that the absorption block may offer at least a partial solution to soil water measurement problems. Other investigators (**1, 8, 10, 11, 13, 16**) have confirmed the shape and general characteristics of this curve, which, it is interesting to note, exhibits a marked resemblance to the moisture stress-soil moisture curves recently developed by Wadleigh (**15**). This resemblance confirms the fundamental nature of the absorption block technique.

Unique features of the absorption block are that (a) the relationship between moisture in the soil and block resistance is basically one of free energy and (b) the measurable range of sensitivity coincides with the

[1] Authorized by the director for publication as journal article No. 955 of the Michigan Agricultural Experiment Station.

[2] Garstka, W. U. Unpublished data. Michigan Hydrologic Research Project, Soil Conservation Service, U. S. Department of Agriculture.

critical variation of soil water. In other words, the range of the technique corresponds with the quantity of moisture between the permanent wilting point and approximately the moisture equivalent. In terms of the absorption block technique, this *available water* is the water held in

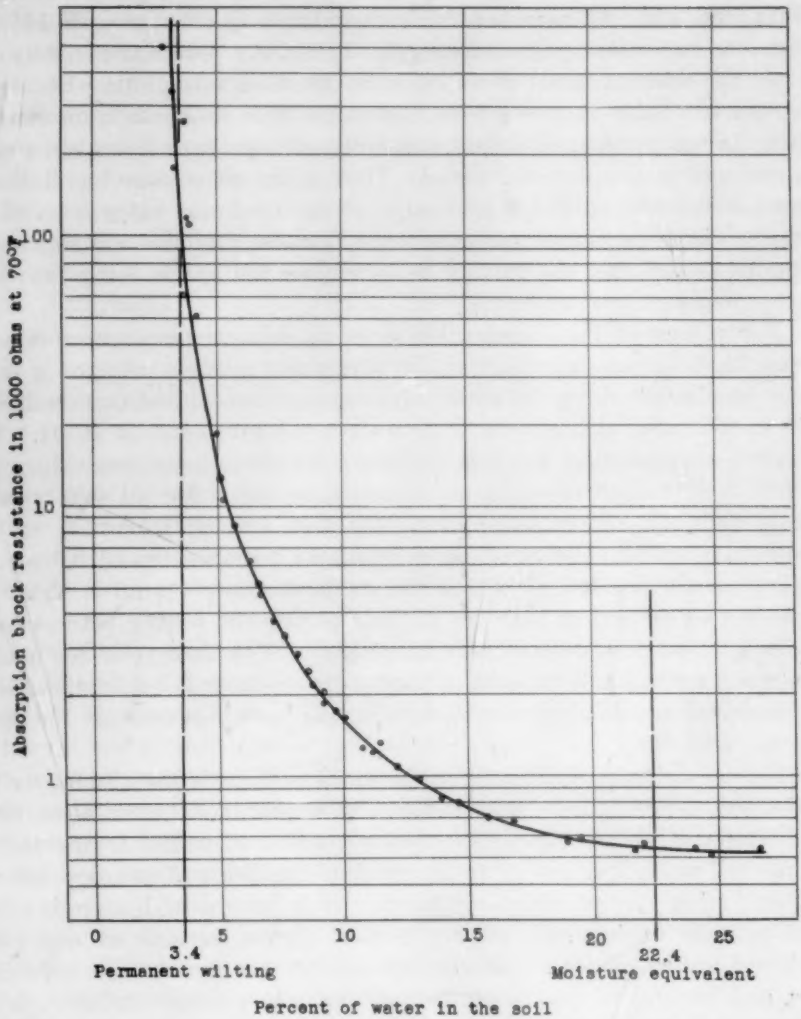

FIG. 1. Curve illustrating the relationship between absorption block resistance and soil moisture.

the soil by forces of such low magnitude that the block resistance is less than the asymptotic values which the curve approaches with respect to the moisture index. An arbitrary average resistance of 75,000 ohms for plaster of Paris absorption blocks has proved practical as an indicator of the maximum forces against which plants can obtain moisture from the soil

(5). The steep gradient of that portion of the curve in question means that exceedingly small changes in the volume of total soil water give rise to relatively great resistance changes. These are of little practical significance, however, because of the small moisture volume changes. Moreover, it is a generally accepted concept that the wilting point (by virtue of its definition) is a narrow range rather than a specific value (**9, 14**), and therefore an arbitrary resistance value becomes a practical necessity.

At the other extreme, when the curve becomes asymptotic with respect to resistance, that is, when block resistance falls to a minimum constant level, the soil contains abundant moisture and is perhaps losing some water in response to gravitational forces. Here again, experience has indicated that a convenient, although arbitrary, critical minimum value is about 600 ohms. Rising resistance values indicate that the available soil moisture is decreasing and that the portion remaining in the soil is being held with correspondingly greater forces.

For plaster of Paris absorption units an arbitrary resistance value of about 75,000 ohms characterizes the permanent wilting point of a fairly large number of widely different soil samples, whereas resistance values of 450 to 650 ohms characterize their moisture equivalents (**2, 3, 5**). This evidence suggests that, for practical purposes, these resistance values represent their respective extremes of moisture status for all soils (except where high salt concentration, and thus high osmotic potentials, are encountered). The validity of these relationships is confirmed by present information which indicates that the soil moisture potential is about the same for all soils when they are at their permanent wilting percentage or at their moisture equivalent percentage (**14**). This feature of the absorption block technique is thought to possess great practical implications, since it eliminates the necessity for calibrating the instrument except for exacting applications.

A more accurate and more fundamental concept is that for any given resistance, different soils hold water with approximately the same force. This statement is comparatively exact when limited to the drying portion of the soil water fluctuation cycle, which is the phase of primary interest in forecasting crop water requirements. It is because of hysteresis exhibited between drying and wetting tension curves, because at any given moisture content different soils do not exhibit the same pressure deficiencies, and because of osmotic differences that an apparent variation is encountered in any attempt to correlate thermodynamic measurements with more or less empirical constants. Statistical significance cannot be expected to obtain, at least not to a high degree, between such attributes. Nor is a high degree of significance necessary, because the moisture constants are of little value in actual field management practices. Despite their inherent inadequacies, however, the constants are here employed to facilitate the presentation of this technique, because they are familiar terms, and because they have been widely, although often erroneously, used.

Detailed studies may be accomplished by calibrating absorption block resistances against soil moisture, correcting all measurements to a standard temperature. Other investigators (10) have perfected a fairly rapid and satisfactory calibration technique which enlarges the scope of this method, adapting it to many agricultural, industrial, and even fundamental research studies involving soil moisture forces, stresses, flows, and also volume and mass relationships. It is emphasized, however, that calibration is not necessary to the use of the standard block resistance as an indicator of the force with which moisture is held by the soil, or as an indicator of approximate volume and mass relationships, that is, the amount of available water present in the soil.

Enhancing the value of the absorption unit resistance as a soil moisture indicator for use in large-scale installations are the following advantages:

1. After the initial installation, which is relatively simple, the soil need not be disturbed.
2. Readings may be made by unskilled labor.
3. Single readings require a minimum of time, generally not in excess of 1 minute; several hundred readings can be made by a single operator in the course of a working day.
4. Absorption units may be completely buried so that their presence does not interfere with surface tillage or plant growth or in any other way with crop production.
5. Absorption units are not costly; this, together with the ease of obtaining individual measurements, makes feasible a large number of replications.

Another advantage of this method is that it gives an indication of the time at which soil freezes, a factor that is sometimes of interest in hydrologic investigations (7, 16). Freezing within the soil mass is indicated by an abrupt rise in the resistance of the absorption unit, caused by a sharp decrease in its conductivity as moisture within it changes from the liquid to the solid state. This transition point cannot be obtained by the use of thermometers, since temperatures considerably below the critical point may not result in freezing within the soil.

Plaster of Paris units

Under Michigan climatic conditions, plaster of Paris blocks have functioned satisfactorily in well-drained soil profiles for more than five years. On the other extreme, waterlogged conditions encountered in low topographic positions reduce their useful life to a single growing season; this is particularly true in organic soils where the $CaSO_4$ dissolves rather rapidly. In intermediate drainage positions, the useful life of the block appears to be determined by the relative length of time it is exposed to saturated conditions. In general, the higher and drier its position, the longer it will function. Freezing conditions do not appear to deteriorate the blocks as rapidly as does solution; considerable physical disintegration,

especially at the corners, is observed after exposure to repeated freezing and thawing, but this superficial disintegration has little effect on the resistance characteristics of the block, unless the volume between the electrodes has been substantially reduced.

Investigations concerning the design of the absorption block have led to the conclusion that the original specifications are indeed practical, although perhaps not so theoretically sound as, for example, a coaxial design. Studies of cylindrical blocks containing concentrically arranged electrodes showed that the effects of external electrical fields were minimized. Difficulties were encountered, however, in casting such blocks with a high degree of uniformity. The relative simplicity of the present rectangular pattern, and the ease and cheapness with which such absorption units can be manufactured appear to offset any theoretical advantages of a coaxial absorption unit. Large quantities of commercially manufactured blocks made according to the original specifications (**4**) include an average of two defective units in every hundred.

Many varieties of material are marketed as plaster of Paris. Differences in both the physical and chemical characteristics of these materials are caused by the varying quantities of hasteners or retarders which are added to control setting speeds and otherwise standardize a given product for commercial and technical uses. In general, these regulating agents have an unfavorable influence on the soil water-resistance relationships of the blocks; chemically pure materials exhibit a wider range of resistance change for a given change in soil moisture and therefore contribute to the sensitivity of the method. Pure gypsum (sold by the United States Gypsum Company as No. 1 White Molding Plaster of Paris) has proved extremely durable and the best material so far investigated. It sets in about 30 minutes and possesses a very porous, although relatively soft, structure.

Two materials of a somewhat similar nature, marketed as Hydrostone and Hydrocal, have been used, because of their extreme hardness, by several investigators. It was thought that this hardness might indicate greater durability and that blocks made of hard materials would last longer when buried in a soil. Experience with these materials has shown, however, that their high density is complemented by a low porosity which reduces not only the relative proportion of water that can be absorbed but also the speed with which it moves within the block and between the block and the soil. The sensitive range of these blocks is narrow, possibly because of the narrow pore-size distribution characteristics. A third objection is the low solubility of these hard materials, which reduces their buffer capacity with respect to salt concentration changes within the soil solution.

Other kinds of absorption units

For the agronomist and other investigators interested in plant-soil relationships, plaster of Paris block appears to be an ideal unit because it covers the range of available water, indicating both the field capacity and

the wilting point of a soil. An absorption unit with a wider range would, however, prove a useful tool especially to the engineer interested in hydrology or construction. An extensive search has therefore been conducted to find absorbent materials with energy characteristics and physical properties superior to those of plaster of Paris, and with the capability of indicating moisture changes up to the saturation point. A large number of clay and cement substances were examined. Also tested were many fibrous materials such as glass fabrics and nylon. Specially designed contact electrodes were re-examined, with the view toward minimizing their former inadequacies.

In Figure 2 are shown six types of resistance units which have given good laboratory performances. With the exception of Unit 5 all of these devices consist essentially of three parts: (1) a porous absorbent (2) electrodes of some resistant metal and (3) well-insulated, weather-proof leads which serve to join the electrodes to a resistance measuring apparatus. These units are dissimilar in that their electrode patterns differ and that their absorbent materials are not alike, either physically or chemically. The construction of these units is briefly described below:

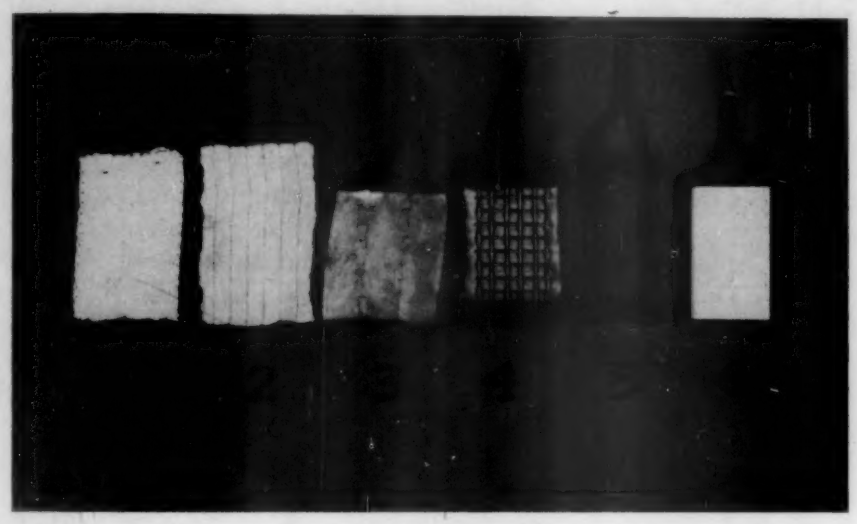

FIG. 2. Six types of experimental absorption units. Their construction is described in the text.

UNIT	ELECTRODES	ABSORBENT
1.	Single-strand monel wires, widely-spaced, and woven into the fabric.	Fiberglas or nylon
2.	Triplicate stainless steel wires arranged alternately in series, and woven into the fabric	Nylon
3.	Medium spaced monel wires stitched to fabric	Nylon or fiberglas
4.	Heavy ¼-inch monel hardware cloth	Nylon or fiberglas
5.	Heavy ¼-inch galvanized iron hardware cloth	None
6.	Multiple-strand tinned lamp cable	Plaster of Paris

In all units but No. 5 the resistance that develops between the electrodes when the unit is imbedded in a drying soil is caused to a large extent by the decrease in conductivity of the porous absorbent. In Unit 5, however,

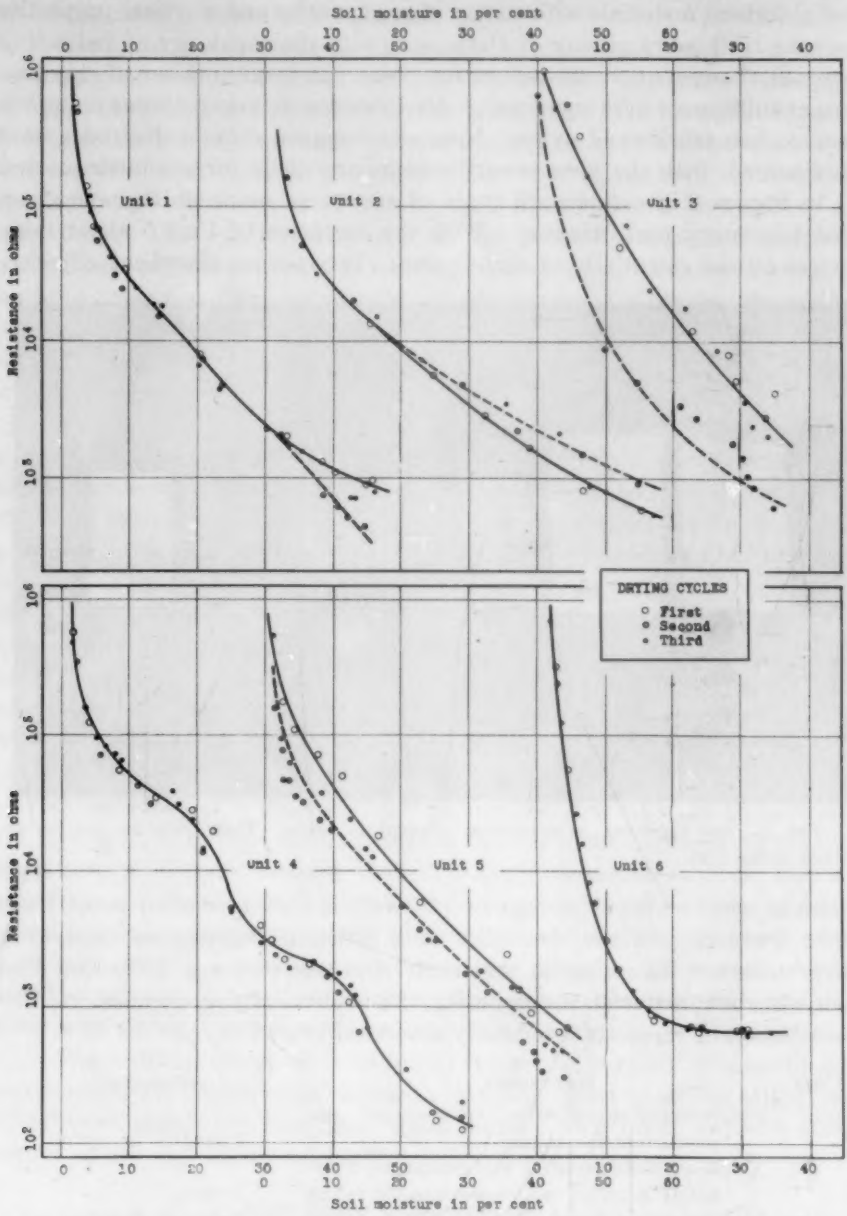

FIG. 3. Typical moisture-resistance curves for the several types of soil moisture measuring units. Moisture indices, in terms of dry weight percentages, are indicated above and below for each of the upper and lower curves, respectively. Unit 6 was imbedded in a light sandy loam, all others were in different kinds of silt loams.

the resistance increases as the soil dries because of decreased conductivity within the soil mass proper. Figure 3 shows a selection of typical laboratory curves obtained with these various units imbedded in different soils. These curves are all similar. They approach minimum values when the soil is at or near saturation. On the opposite extreme they approach maximum values at minimum moistures. Depending on the pore-size distribution of the absorbent materials all of these units possess the capability of yielding a moisture index over a wide or narrow range. With the exception of Unit 6 all units are sensitive nearly to the point of saturation.

It has already been pointed out that Unit 6 is unique in that its curve yields a close approximation of the permanent wilting per cent. and field capacity. Unit 4 is of special interest because it appears to operate over an extremely wide moisture range. Preliminary tests indicate that it yields fairly accurate and reproducible results from air-dry conditions to saturation and for this reason it is being developed and refined. Because experimental errors cannot be ignored, the range of these curves is only one aspect of their utility. Unit 6, the standard plaster of Paris block, although operating over the smallest range, exhibits the smallest scatter of items within its range. Not only does this unit show the smallest experimental error within a single drying cycle, but it also shows the least hysteriesis between successive drying cycles, thus illustrating the unique buffering function of its slightly soluble gypsum absorbent.

Despite the advantage of being responsive over a wide range of moisture conditions, units other than 6 possess certain inherent disadvantages in addition to their relatively large experimental errors. Pressure changes resulting from swelling and shrinkage not only contribute to these inherent errors but they may, under extreme conditions of dryness, result in a separation of the absorbent from the soil matrix which interrupts the operation of the unit. Plaster of Paris units not only withstand these pressure changes but they will continue to function as long as a small area of contact remains between the absorbent wall and the soil. The plaster of Paris units are therefore favored, especially where plant-soil relationships are involved, because they permit more quantitative measurements than do the other types, even though the latter are more sensitive to moisture changes. Where it is desirable to measure the entire range of moisture conditions, from the air-dry state to saturation, then Unit 4 is recommended. A careful application of this technique will yield satisfactory results.

The resistance bridge

A major obstacle in the development of a practical procedure for field use was the lack of a satisfactory resistance measuring device. Commercial meters already on the market possessed many disadvantages chief of which were a limited range, delicate construction, bulkiness, and high cost. In order to overcome these obstacles and to benefit from recent advances in instrumentation it was necessary to construct a special resistance bridge.

As now manufactured, this improved bridge combines rugged, compact construction with a high degree of sensitivity. Both the resistance bridge and the plaster of Paris blocks are manufactured and sold by The Wood and Metal Products Company, Bloomfield Hills, Michigan. The improved

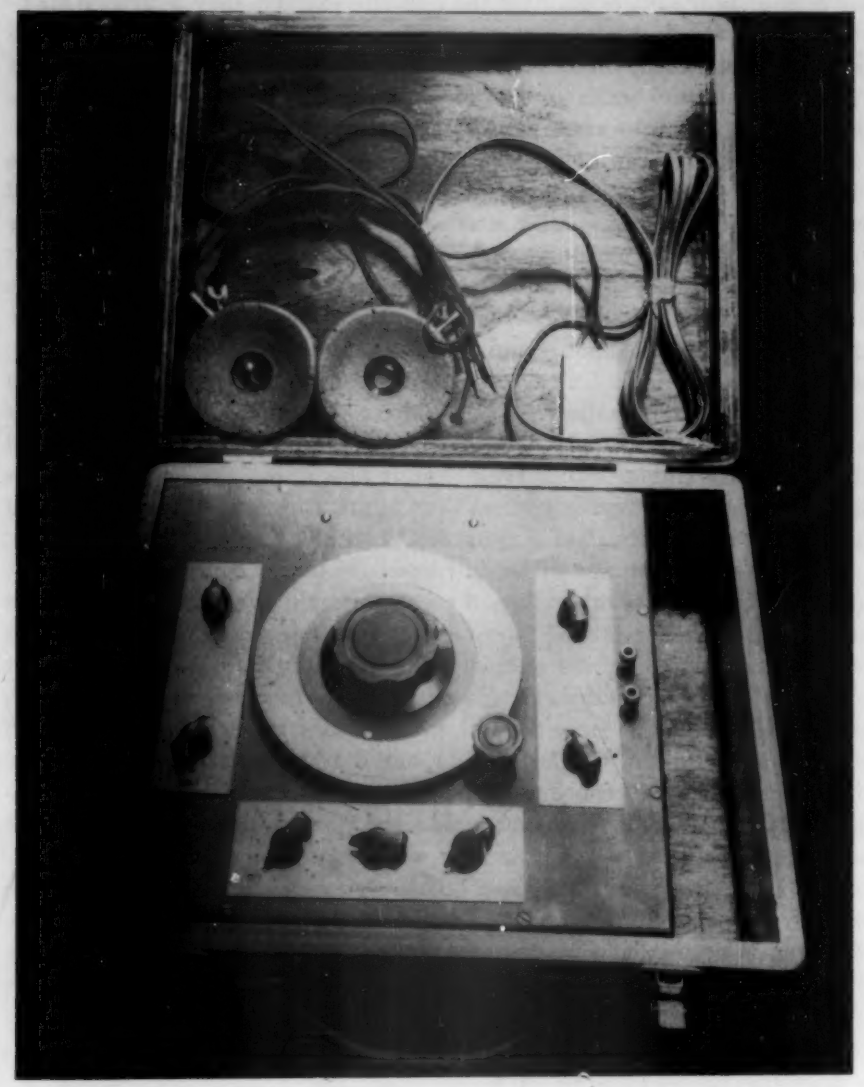

FIG. 4. The resistance bridge used with the absorption units described. The unit is self-contained and easily portable, weighing about 20 pounds.

bridge is a completely self-contained unit (Fig. 4) which is designed to measure resistances in circuits containing considerable capacitance, such as is encountered in installations where the blocks may be connected by up to 200 feet of commercial multiple-strand rubber-coated copper wires.

Experience with many designs has indicated that a wooden case helps to shield the instrument from ground currents, a factor in metal-cased instruments that invariably causes difficulties under certain combinations of circumstances frequently encountered in the field. Headphones are greatly preferred to an "electric eye" indicator. The electric eye, although a satisfactory null indicator in the laboratory, is difficult to read under field light unless a shadowbox or curtain is used. This increases the bulkiness of the instrument and adds to the difficulties of manipulation. Galvanometers, likewise, proved unsatisfactory for general field use, inexpensive commercial models being too delicate or else, on the other extreme, lacking in sensitivity. A relatively inexpensive headphone set, however, has proved durable and efficient for general outdoor use. Making use of the sound characteristics of the necessity oscillating current, headphones have proved to be the most useful type of null indicator. In conjunction with the present circuit design, a great contrast in tone volume, which rapidly fades to a minimum level within an extremely narrow range, contributes to the ease of adjusting the instrument. For prolonged operation, sponge rubber cushions over the phones have been found to be helpful by reducing the interference of extraneous sounds such as are produced by wind currents. They also distribute the mechanical pressure over the entire ear cartilage, which adds to the comfort of the operator.

The circuits of the improved bridge are based on the Wheatstone principle. To avoid the influence of various capacitance factors present in field installations, a large condenser has been included which contributes greatly in obtaining a good null balance within the bridge. The instrument is powered by dry-cell batteries feeding through a 2000-cycle electronic oscillator. An extremely wide range of sensitivity is obtained by inserting two series of standardized resistances in opposite arms of the bridge; the proper combination is selected by means of multiplier switches, and the final null-point is obtained by adjusting a logarithmic potentiometric rheostat fitted with a 6-inch graduated dial. The bridge is thus balanced, or tuned to the null point, by manipulating five dials in a matter of seconds. An ohm-meter modification of this circuit has been successfully used for several seasons to control irrigation on experimental fields. When the absorption block leads are plugged into this moisture meter, movement of a needle across the dial indicates the extent to which soil moisture has been depleted.

Summary

1. The electrical resistance technique of obtaining a continuous measure of soil moisture *in situ* under field conditions by means of absorption units is discussed in the light of additional knowledge and experience gained since the inception of the method in 1940.

2. Fundamental considerations of the characteristics of this technique reveal that for most practical purposes standard plaster of Paris absorption blocks need not be calibrated. Resistance readings may be directly

interpreted in terms of available soil water; in all soils the percentage of available water is approximately the same for any given resistance value.

3. Several types of adsorption units are described and compared by means of laboratory calibration curves. Some of these units offer great promise of measuring soil moisture from saturation to dryness.

4. The advantages of the method are summarized.

DEPARTMENT OF SOIL SCIENCE
MICHIGAN STATE COLLEGE
EAST LANSING, MICHIGAN

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RESPONSE OF KALANCHOE TUBIFLORA TO X-RADIATION

EDNA L. JOHNSON

(WITH SIX FIGURES)

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A study extending over three and one-half years was made of the vegetative and reproductive growth of *Kalanchoe tubiflora*, (Harvey) Hamet., a Madagascan succulent of the Crassulaceae. This plant, which grows well in greenhouses of this country particularly during the hot days of late spring and summer, is well adapted to a study of responses to various environmental factors. Studies (**2**) have been made of its response to different light intensities, different qualities of light as supplied by fluorescent light tubes, supplemental artificial light, and to X-radiation. The present paper will be limited to the response to X-radiation.

Plants of this species of Kalanchoe were first seen by the writer in the gardens of the Atkins Institution of the Arnold Arboretum at (Soledad) Cienfuegos, Cuba. The present superintendent, Mr. Frank Walsingham, suggested their probable value for experimental work and made it possible to secure some young plants from Mr. A. C. Jordahn, then associate superintendent of the Fairchild Tropical Garden at Cocoanut Grove, Florida. From these original plants, hundreds have been grown in the University of Colorado greenhouse.

Kalanchoe tubiflora has a long slender unbranched stem with spirally arranged leaves. However, vegetative shoots which arise in the region of the terminal inflorescence after the flowers have faded, give old plants a branched appearance. The almost cylindrical leaves which are 4–5 mm. wide and 6–7.5 mm. long are normally strikingly mottled with purple streaks or blotches. Typically the leaf tip has from four to seven small teeth. When the plant is reproducing vegetatively, protuberances extend outward from between the teeth. CLAMP (**1**) refers to the protuberances as leaf claws. At the tip of each claw, develop little plantlets each consisting of a short disc-like stem bearing two pairs of leaves (Fig. 1). While in this two-leaved condition, the plantlets fall to the ground and develop into new individual plants.

Methods and material

Plants of different ages were used, but this paper will deal principally with a group composed of tiny plantlets which drop from the older plants. The growth of these, however, will be compared with that of three other groups which were irradiated in the 8-leaved, 15-leaved, and 30-leaved stages respectively. The study was begun during the latter part of November and final records were taken 3½ years later. Tiny plantlets were irradiated with 2500 r-units of X-rays; these with an equal number of controls were placed

in pots in the greenhouse. Five months later they were dug, washed, the leaves counted, and the plants weighed. The group of 137 experimental plants weighed slightly more than an equal number of controls. The amount of water displaced by the irradiated group was 5.5 per cent. greater than that displaced by the controls. The average number of leaves on the experimental plants was 13.5 per cent. greater than on the controls.

Before replanting, the experimental group of plants was again irradiated with a second dose of 2500 r-units. Although both controls and experi-

FIG. 1. Plantlets seen at the tips of the mottled leaves of *Kalanchoe tubiflora*. (Courtesy of New York Botanical Garden.)

mental plants were carefully handled during the leaf counting and weighing, quite a few died soon after they were replanted in the soil. It is of interest to note that the controls at this age seemed less hardy than the experimental plants, for 27.7 per cent. of them died following this procedure as contrasted with 14.6 per cent. of the irradiated. The control group at this time numbered 99; the experimental group, 117.

Results and discussion

VEGETATIVE GROWTH.—Records of vegetative growth taken at intervals

up to the time of flowering are given in table I. The number of leaves which bore plantlets was considerably increased in the experimental group as shown by records taken eight months after treatment. The increase of the experimental over the control was as great as 47 per cent. 17½ months after treatment.

The number of leaves reproducing by bearing tiny plantlets gives some index to the vegetative vigor of the parent plant as does the number of plantlets present. The numbers recorded are those which were on the plants the day the records were taken. No attempt was made to estimate the total number of plantlets formed as they were constantly developing and then soon dropping to the ground. The number of plantlets present on the plant

TABLE I

VEGETATVE GROWTH OF *Kalanchoe tubiflora* (117 PLANTS GIVEN TWO DOSES OF 2500 r-UNITS OF X-RAYS; CONTROL, 99 PLANTS)

	AGE IN MONTHS	CONTROL	IRRADIATED	INCREASE OF IRRADIATED OVER CONTROL
				%
Av. no. of leaves per plant	5 (April)	11.9	13.6	13.5
Av. no. of plantlet-bearing leaves	6 (May)	2.4	2.1	−12.0
	8 (July)	12.6	13.5	7.1
	11 (Oct.)	5.8	7.7	32.7
	17.5 (May)	18.0	26.2	47.2
Av. no. of plantlets	6	4.2	3.6	−14.3
	8	43.7	42.6	− 2.5
	11	10.9	14.0	28.4
	17.5	85.7	98.2	14.6
Height to plant tip (cm.)	8	10.3	10.4	1.0
	11	18.4	18.7	1.6
	14.5	20.4	21.6	5.9
	17.5	30.3	33.3	9.0

varied a good deal with the age of the parent plants and particularly with the season. During the long, warm days of late spring and summer, they were especially abundant as shown by the records taken the eighth month of the experiment, which was in July. Actual counts made of the number of plantlets indicate a considerable increase in the irradiated group over that of the control at the end of the eleventh and seventeenth months. Heights of the irradiated plants exceeded those of the controls at every measurement made during the first 17 months; however, there was not a great deal of difference between the two groups.

INDUCTION OF BRANCHING.—One conspicuous response to irradiation was the induction of branching. Records taken 17½ months after treatment gave a total of 20 per cent. branched forms among the experimental plants while all of the 99 controls remained single-stemmed. The time when

branching occurred is graphically shown in figure 2. Of the total branching, only 4.3 per cent. was evident at the end of six months; 26.1 per cent. more during the following two months. At the end of the eleventh month, 43.4 per cent. more was recorded; between the eleventh and fourteenth months, 13 per cent. more had branched while as late as 17.5 months after treatment, an additional 13 per cent. of those which branched, displayed this condition.

The region of the stem where branching occurred varied considerably.

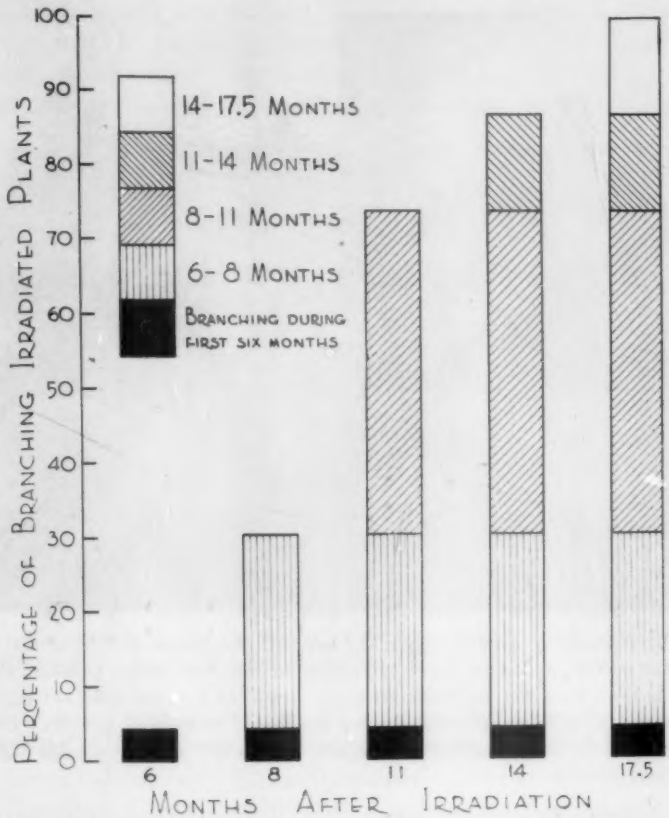

FIG. 2. Chart represents time when branching occurred in plants which were X-rayed with two doses of 2500 r-units in the plantlet stage. Note that the highest percentage of branching occurred about a year after treatment; 13 per cent. branched as late as between 14–17.5 months after irradiation.

Thirty-five per cent. branched at or near the base; 8.7 per cent. at a height not exceeding 10 cm. from the base; 47.7 per cent. at a height between 10 and 20 cm., while the remaining 8.7 per cent. branched higher than 20 cm. from the base. Of the total number branching, all but three branched dichotomously; one of these branched at the base, and later one of the branches forked at a height of 9 cm. A second plant branched at the base,

then each branch forked again, one at a height of 5.5 cm. and another at a height of 11 cm. A third plant which branched at a height of 10 cm., branched again at 15 cm. Different types of branching are shown in figure 3.

REPRODUCTIVE DEVELOPMENT.—*Kalanchoe tubiflora* is a short-day plant as it flowers only during the short days of the winter months. The drooping flowers which are red-orange in color occur in many-flowered terminal paniculate cymes, rather large and showy. The green calyx is four-parted with

FIG. 3. Kalanchoe plants. Left, 2 normal single-stemmed controls; right, 6 experimental plants typical of those irradiated in the very young plantlet stage. The first plant to the left in the experimental group branched in three different regions. The first branching occurred at the base during the first 6 months of growth. By the 11th month, each branch had forked again, the left at a height of 5.5 cm.; the right, 11 cm. above the first division.

narrow acuminate pink-tipped lobes which are considerably shorter than the corolla-tube; the four-parted corolla tube, which is urn-shaped with spreading lobes, is approximately four cm. in length. The eight stamens are adnate to the base of the corolla tube; the carpels are four in number. That the blossoms remain in good condition for a considerable period is shown by the fact that the earliest blooming plant of the 127 which blossomed the second winter, produced its first flower on November 23; complete records for the reproductive structures which were taken 51 days later gave for this same plant a total of 45 fresh flowers and 37 buds. Figure 4 represents a portion of the plants which bloomed during their second winter of growth.

The earliest time of flowering for any of the plants was 724 days after treatment when an irradiated plant came into blossom; two days later the first control blossomed. It is significant that 23 per cent. of the irradiated plants flowered in less than two years, while but 11 per cent. of the controls had blossomed in a similar period. However, when the average time was calculated for the blooming period of those which blossomed during the second winter, it was found to be 735.8 days for the irradiated and but one day longer for the controls.

Data on reproductive development will be given separately for the group which blossomed at the approximate age of two years and for a second group made up of the remaining plants which did not flower until the third winter. Records for the total number of reproductive structures of the former group

FIG. 4. *Kalanchoe tubiflora* flowering in the University of Colorado greenhouse. The photograph was taken during the second winter when a total of 127 plants flowered; 68 of the remainder blossomed the following winter.

show them to be slightly fewer for the experimental plants than for the controls (table II). However, the higher percentage of faded flowers and the lower percentage of buds on the irradiated plants clearly indicate that there was a general trend toward earlier maturity of the treated plants. It will be noted from table II that this first group of blossoming plants composed 65.3 per cent. of the controls and 54.3 per cent. of the irradiated plants. It is evident that branching of those exposed to irradiation had delayed flowering of the group as a whole. Contrasting the percentage of the unbranched flowering experimental plants with the controls will give a more accurate conception of the effect of irradiation upon reproduction. The percentage of unbranched irradiated plants blooming at this time was 63.8 per cent. as contrasted with 65.3 per cent. for the controls.

After a study was made of the shoot development in the inflorescence region and records of total height and fresh weight taken, the plants which had blossomed were discarded. There remained one-third of the original

TABLE II

BLOSSOMING OF *Kalanchoe tubiflora*

	PLANTS IN BLOSSOM		TOTAL NO. OF REPRODUCTIVE STRUCTURES		CONDITION OF FLOWERS						AV. TIME FOR BLOSSOMING (DAYS)	NO. BLOSSOMING BEFORE 2-YR. PERIOD (%)
					FADED, BUT ATTACHED		FRESH, BUT MATURE		BUDS			
	No.	% OF TOTAL	No.	Av.	No.	%	No.	%	No.	%		
GROUP BLOSSOMING DURING SECOND WINTER												
Control	64	65.3	5231	81.7	2526	48.3	1283	24.5	1422	27.2	736.8	10.9
Irrad.	63	54.3	4862	77.2	2508	51.6	1293	26.6	1061	21.8	735.8	22.6
GROUP NOT BLOSSOMING UNTIL THIRD WINTER												
Control	26	28.4	.....	.....	.....	.....	.....	.....	.....	.....	1112.8	.....
Irrad.	42	40.2	.....	.....	.....	.....	.....	.....	.....	.....	1112.1	.....

number of controls and 45 per cent. of the original irradiated plants to be observed during the third year of the experiment. During the third winter, 26 more controls blossomed and 42 more of the irradiated forms. Figure 5 shows the typical appearance of blossoming controls and experimental branched plants. Thirty-six per cent. of the branched plants which flowered

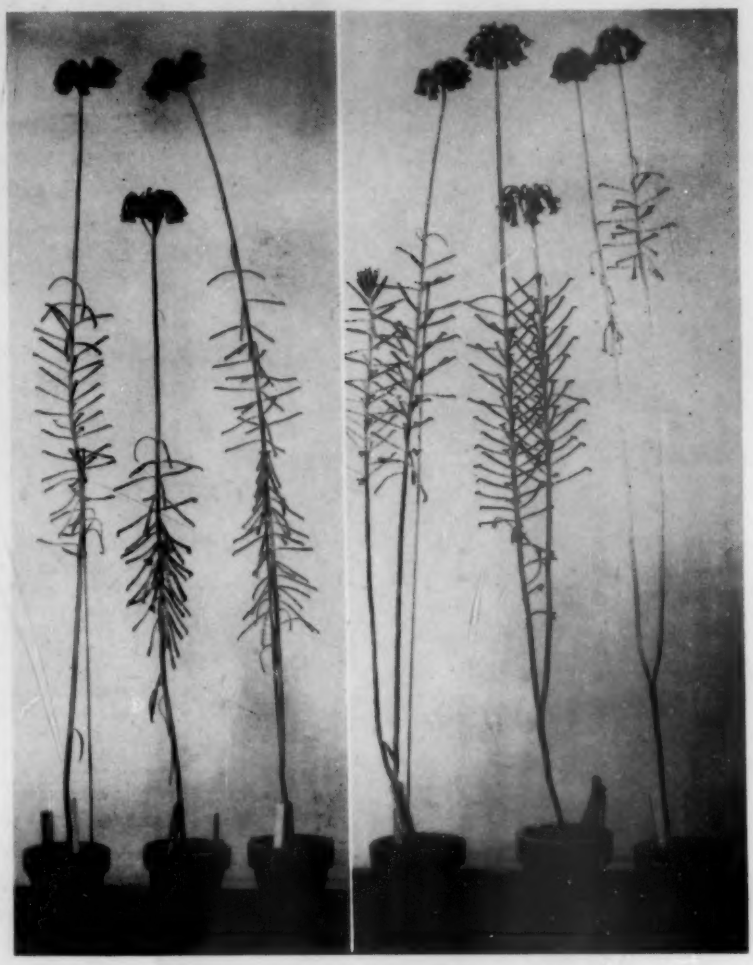

FIG. 5. TYPICAL BLOSSOMING controls and experimental branched plants of *Kalanchoe tubiflora*. Thirty-six per cent of the branched plants which flowered within the 3½-year period of the experiment bore flowers on two shoots.

within the 3½-year period of the experiment had flowers on two shoots. Data given in table II indicate little difference in the blossoming behavior of the control and experimental groups in the average number of days required for blossoming and in total number flowering.

SHOOT DEVELOPMENT IN REGION OF OLD INFLORESCENCES.—When the

flowers of this species of Kalanchoe begin to fade and drop off, shoots with very small leaves develop in the inflorescence region. Records taken of the reproductive structures of the group blossoming the second winter, indicated

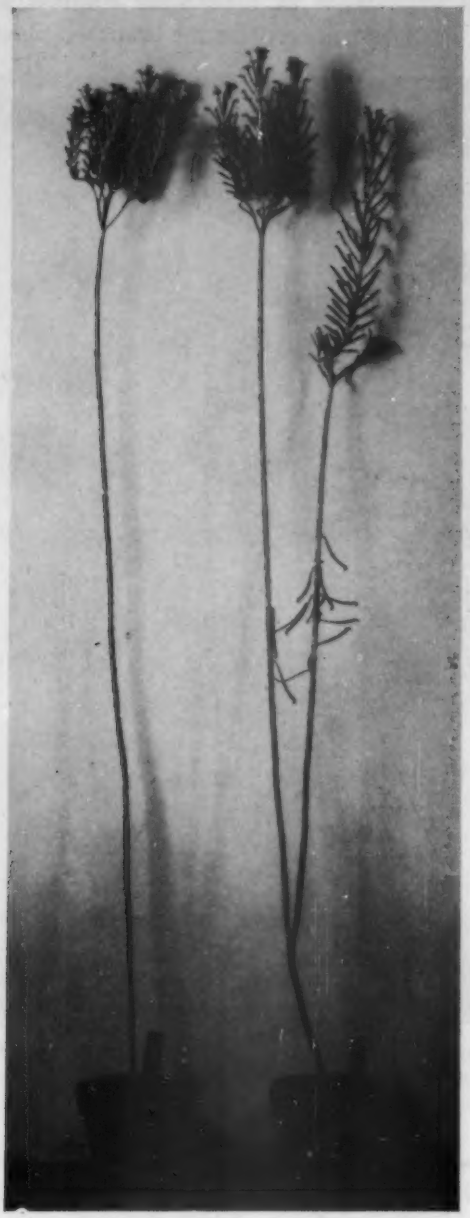

FIG. 6. View of terminal tips of Kalanchoe plants showing shoots developing in the region of the inflorescence. Left, typical control; right, one of the branching irradiated plants.

that the average number of shoots which had developed at this time in the terminal regions of the flowering irradiated plants was 37 per cent. greater than in the controls. This indicates earlier maturing rather than stimulation of branch formation, for at the age of 30 months the average number of terminal shoots in the irradiated plants was but 0.4 per cent. greater than in the controls. Figure 6 gives a view of typical plants after shoots in the inflorescence region had made considerable growth.

In the group which did not flower until the third winter so that records were not secured until the plants were 43 months of age, the average number of terminal shoots on the irradiated plants was 31.8 per cent. greater and the average total shoot length per plant was 9 per cent. more than in the controls. Vegetative growth of irradiated plants as determined by height

TABLE III

VEGETATIVE GROWTH AFTER FLOWERING PERIOD

	CONTROL			IRRADIATED		
	GROUPS FLOWERING SECOND WINTER		FLOWERING THIRD WINTER	GROUPS FLOWERING SECOND WINTER		FLOWERING THIRD WINTER
	AGE IN MONTHS			AGE IN MONTHS		
	30	33	43	30	33	43
Av. height (cm.)	135.5	142.8	179.8	133.9	141.6	168.0
Av. no. of terminal shoots	9.2	.....	6.6	9.6	.....	8.7
Av. total shoot length per plant (cm.)	128.6	.....	71.0	119.7	.....	77.4
Av. no. of plantlets in terminal shoots	115.1	.....	.....	103.8	.....	.....
Av. fresh weight (gm.)	.....	118.4	142.7	.....	112.4	138.6

was increased over the controls until the time of flowering. After that time, however, when the total height measurement was made to the tip of the terminal shoots forming in the inflorescence region, height measured in the group which flowered the second winter and also in those not flowering until a year later, indicated that the irradiated plants did not average as tall as the controls.

The plants flowering the second winter were cut down and the above-ground parts weighed 33 months after the experiment was begun; green weight was not determined for the group flowering the third winter until ten months later. In both cases, the controls weighed slightly more than did those of the experimental groups (table III).

EFFECT OF IRRADIATION UPON OLDER KALANCHOE PLANTS.—Groups of plants averaging 8, 15, and 30 leaves each were irradiated with 2500 r-units in order to determine the effect of irradiation upon plants older than those

used in the experiments just described. Thirty-three plants were used in the 8-leaved group, 24 in the 15-leaved group, and 19 in the oldest lot. Records taken two months and again three months after treatment, indicated increased leaf number over that of the control in the group irradiated in the 8-leaved stage; there was no increase in this respect in the other groups. From a study of table IV which summarizes the vegetative development of the three groups for a period extending from 5 to 17.5 months after irradiation, it is clear that any stimulation occurring from the treatment was evident only in the youngest group.

TABLE IV

VEGETATIVE GROWTH OF GROUPS OF KALANCHOE PLANTS WITH 8–30 LEAVES AT TIME OF IRRADIATION

	CONTROL	IRRADIATED	CONTROL	IRRADIATED	CONTROL	IRRADIATED
	8-LEAVED GROUP		15-LEAVED GROUP		30-LEAVED GROUP	
	AV. NO. OF PLANTLET-BEARING LEAVES PER PLANT					
Time after exp. was begun (months)						
5	2.4	1.0	1.7	1.7	1.2	1.4
6	13.7	14.6	13.7	14.2	11.4	11.0
8	25.7	26.1	27.4	26.9	24.0	25.2
11	21.1	22.0	19.2	19.4	29.9	29.7
17.5	9.2	9.4	9.7	9.5	17.3	16.3
	AV. NO. OF PLANTLETS PER PLANT					
5	3.8	1.8	2.8	2.2	2.1	2.4
6	50.0	45.5	44.9	42.0	36.4	34.9
8	97.3	94.4	111.0	100.4	96.9	95.4
11	51.8	51.0	46.0	44.3	75.1	82.8
17.5	22.7	24.5	24.7	22.8	45.7	48.1
	HEIGHT TO TIP OF PLANT (CM.)					
5	5.7	5.1	7.8	7.5	18.0	16.5
8	23.6	21.2	26.5	25.6	35.5	32.0
11	36.2	34.5	40.0	39.0	56.4	47.1
14.5	41.	35.0	43.0	42.2	65.0	51.5
17.5	47.1	39.7	50.5	47.4	73.6	61.5

The number of plantlet-bearing leaves in the irradiated youngest group was increased over the controls as shown by records taken the 6, 8, 11, and 17.5 months; the average number of plantlets per plant was greater in the treated plants 17.5 months after irradiation. There was not sufficient difference between the control and experimental plants in the two older groups to indicate that the irradiation had much effect on their growth and development. In none of the three groups, at any time when height measurements were made, did the experimental plants exceed the controls.

Branching, which can be induced by irradiating very young plants, was apparent in 23 per cent. of the group in which the leaves averaged eight

in number at the time of irradiation; it occurred in only eight per cent. of the plants which were treated in the 15-leaved stage. The older plants which averaged 30 leaves at the time of treatment did not branch at all. Branching never occurs in the controls.

Many of the plants of the three groups did not blossom during the 17½ months that records were taken; had they been kept until the following winter, the per cent. flowering undoubtedly would have been greater. In the youngest group, 12.5 per cent. of the controls blossomed, none of the irradiated; in the group whose leaves averaged 15 at the time of irradiation, the control did not blossom and but 8 per cent. of the irradiated plants. In the group which was oldest when irradiated, 66 per cent. of the controls blossomed and but 10 per cent. of the treated plants; the latter group, however, blossomed in a shorter time.

COMPARISON OF RESULTS OBTAINED WITH IRRADIATION OF VERY TINY PLANTLETS AND THOSE IN EIGHT TO THIRTY-LEAVED STAGE.—In both the group irradiated when plantlets were very young and those which were in the eight-leaved stage, the number of plantlet-bearing leaves was increased over the controls as shown by records taken during the 8, 11, and 17.5 months after treatment. Counts of plantlets attached to mother plants were larger in the irradiated group than in the controls at the end of 17.5 months in both those irradiated when very young and also in those treated in the eight-leaved stage. While the group which was irradiated in the tiny plantlet stage exceeded the controls in height up to a period of 17.5 months after treatment, those irradiated in the eight-leaved stage never averaged taller than the controls. At no period during the 17.5 months of observation, did the plants irradiated in the 15 and 30-leaved stage have increased number of plantlet-bearing leaves, greater plantlet production, or increased height as compared with the controls. Branching, which occurred in approximately 20 per cent. of both the group treated in the plantlet stage and in the eight-leaved group, was much less evident in the group irradiated in the fifteen-leaved stage, and did not occur at all in those irradiated when older. The above facts clearly indicate that vegetative growth and reproduction, as well as induced branching, are more noticeably affected when Kalanchoe plants are irradiated when very young, less so when in the eight-leaved stage, and scarcely at all when older plants are treated.

Summary

1. Tiny plantlets which drop from the leaves of *Kalanchoe tubiflora*, were X-radiated and records made of the vegetative and reproductive development of 216 plants for a period of three and one-half years.

2. During the first 17 months stimulation was shown by the irradiated plants as evidenced by increases in number of plantlet-bearing leaves, number of plantlets, and greater plant height.

3. X-radiation induced branching in 20 per cent. of the treated plants while none occurred in the controls. Most of the branching became evident

between the eighth and eleventh months of growth; while many of the plants branched dichotomously near the base, a higher percentage forked at a height between 10 and 20 centimeters.

4. The earliest blooming of this species, which is a short-day plant, occurred in an irradiated plant within 724 days after planting. Twenty-three per cent. of the irradiated group flowered in less than two years, while but 11 per cent. of the controls blossomed in a similar period. Considering flowering of the irradiated group as a whole, however, it was found that branching in this group had delayed the time of blooming.

5. Small-leaved shoots develop in the inflorescence region of this species as the flowers fade and drop off. Earlier maturity of experimental plants as determined by shoot development, was indicated by the presence of increased number of developing shoots in the experimental plants as shown by records taken seven weeks after the first blossoms appeared. In the irradiated group which did not flower until the third winter, records taken when the plants were $3\frac{1}{2}$ years old gave a 30 per cent. increased terminal shoot development over the controls.

6. Height of irradiated plants was greater than the controls until time of flowering. Final measurements, however, which were made to the tip of the terminal shoots forming in the inflorescence regions, indicated that the irradiated plants did not average as tall as the control nor was there much difference in the green weight of the two groups.

7. Groups of plants averaging 8, 15, and 30 leaves each were irradiated with 2500 r-units in order to determine the response to radiation of plants older than those in the tiny plantlet stage. Evidence is presented to show that vegetative growth and reproduction, as well as induction of branching, are increased only when very young plants and those up to and including the 8-leaved stage are irradiated.

DEPARTMENT OF BIOLOGY
UNIVERSITY OF COLORADO
BOULDER, COLORADO

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A MICROCHEMICAL EXAMINATION OF McINTOSH APPLE LEAVES SHOWING RELATIONSHIP OF CELL WALL CONSTITUENTS TO PENETRATION OF SPRAY SOLUTIONS

E. A. ROBERTS,[1] M. D. SOUTHWICK,[1] AND D. H. PALMITER[2]

(WITH THREE FIGURES)

Received August 1, 1947

Spray solutions containing minor elements, nitrogen, "hormones" and organic fungicides have been applied as foliage sprays to apple trees to reduce nutritional deficiencies, prevent fruit drop or increase resistance to disease. The fact that such applications have given results showed that they had penetrated the leaves and raised the question of how penetration occurred.

The quality, quantity and location of wall substances throughout the apple leaf and the part these play in the entrance of materials applied as sprays, either as fungicides or nutrient elements, has not been reported other than the preliminary reports of the authors (**5**) and (**6**).

WYLIE (**7**) found that the epidermis is important for water conduction in areas between veins. In experimenting with dilute solutions of potassium ferrocyanide, he showed that it was quickly conducted throughout the epidermal layers of leaves and that the "vein extensions" and epidermal layers "constitute in some degree a supplementary conductive system." MACDANIELS and COWART (**3**) claim that "the outer cell walls" of the epidermis of apple leaves "are covered with a continuous cuticle." While this would not prevent the lateral movement of materials after once having entered the leaf, it would not account for the entrance of foliage sprays as is indicated by HAMILTON, PALMITER and ANDERSON (**2**) and PALMITER (**4**).

In this investigation, a determination of the quantity and location of cutin, cellulose and pectinaceous substances of the cell walls was made by the following methods: Sections were cut from fresh McIntosh apple leaf material, using a freezing microtome. All photomicrographs were made by the use of a Leica Camera attached to a Leitz microscope by means of an Ibso attachment with correction lens giving an initial magnification of $\frac{1}{3}\times$ Periplan $10\times$ eyepiece and a water immersion or other objective. Kodachrome K-135A artificial light film was used. Methods of microchemical determination of the cutin, cellulose and pectinaceous substances were those given in Eckerson's "Microchemistry" (**1**).

The structure of the untreated walls of the epidermal cells (fig. 1) is particulate in character. It shows clearly that these particles are in layers in the outer and vertical walls.

[1] Department of Plant Science, Vassar College, Poughkeepsie, New York.
[2] New York State Agricultural Experiment Station, Geneva, New York.

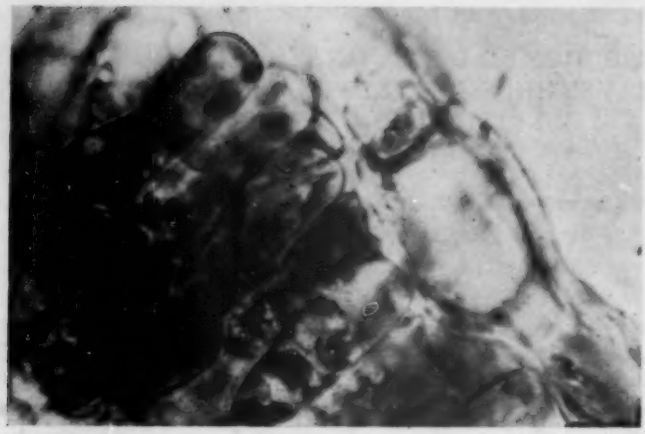

FIG. 1. Cross section of epidermal cells of leaf showing its particulate nature.

In both a surface view of the outer wall of the epidermal cells (fig. 2) and in the cross section, the particulate character and layering of the particles in the walls are shown. The inner portions of the walls, which are the more recently formed, show much irregularity in the layering. This layering shows that the wall is by no means a uniform, solid structure.

The location of cutin, a substance which is known to prevent the absorption of water, was determined by the use of Sudan III, which gives a red color reaction with this substance. A cross-section of a leaf treated with Sudan III shows that cutin exists in lamellae parallel to the outer epidermal walls, but it by no means forms a continuous covering (fig. 3A). Sections parallel to the surface showed the walls with their cutinized areas in discontinuous lines.

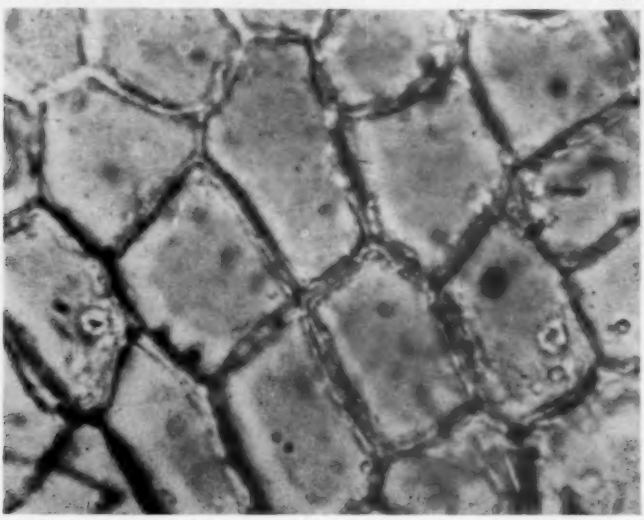

FIG. 2. Surface view of outer wall of epidermal cells showing layering of particles.

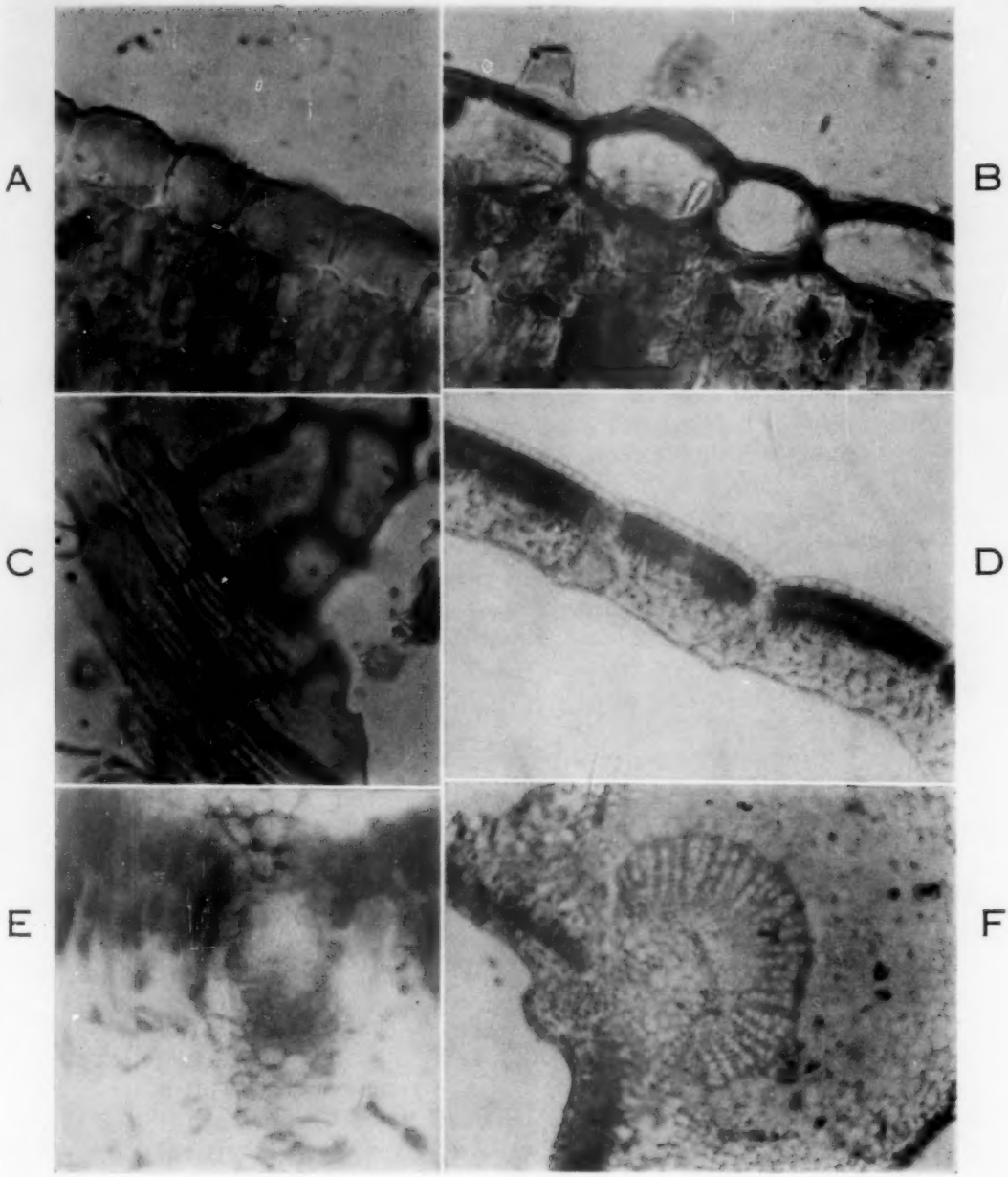

FIG. 3. Tissue sections. A. Cross section showing cutin present in lamellae parallel to outer walls of epidermal cells. B. Cross section of epidermal cell treated with ruthenium red showing location of pectinaceous substances. C. Wall treated as in B showing pectinaceous substance in intermittent strands. D. Cross section treated with ruthenium red, showing pectinaceous substances forming continuous path from outside to walls of "vein extensions." E. Showing amount of pectinaceous substances in walls of vein extension cells. F. Shows large amount of pectinaceous substances in walls of cells of bundle sheath of large vein.

Since the pectinaceous substances are known to have great water absorption power, tests for these substances in sections corresponding to figures 1 and 2 were made. In the presence of Ruthenium red, the pectinaceous substances give a reddish pink color. B and C of figure 3 are cross and parallel sections respectively of the epidermal cells, which show clearly the presence of the pectinaceous substances in all the walls of the epidermal cells. There is much of these substances in intermittent parallel layers in the outer walls of the epidermal cells interspersed with cutinized areas. Layers of these substances extend vertically, whereas the cutinized areas were only parallel. The pectinaceous substances form a continuous path reaching from the outside of the leaf and extending to the walls of the "vein extensions" (fig. 3D).

The walls of the vein extension cells (fig. 3E) contain a large amount of pectinaceous materials as well as do the walls of the cells of the bundle sheath of a large vein (fig. 3F). These are composed largely of pectinaceous substances.

The epidermal cell walls of the McIntosh apple leaf, therefore, can no longer be considered as covered with a continuous cuticle which prevents the absorption of water. The amount and location of pectinaceous substances present in the leaves account for the entrance of water soluble materials such as minor elements, nitrogen, hormones, and organic fungicides sprayed upon apple trees.

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SOIL TEMPERATURE AS A FACTOR IN THE FRENCHING OF TOBACCO (*NICOTIANA TABACUM* L.) [1]

LINUS H. JONES

(WITH FOUR FIGURES)

Received February 18, 1948

During a study of the effect of soil temperature on the development of a tobacco mosaic, frenching was observed on the plants at the higher soil temperatures. Since this is apparently the first time, JONES (**3**), that soil temperature has been recognized as a factor in inducing frenching of tobacco, *Nicotiana Tabacum* L., it seemed important that the technique be developed as a possible means of discovering the direct cause of this peculiar physiological malady.

Tobacco plants grown at the high soil temperature, 35° C., always became frenched, characterized by chlorotic, narrow leaves with wavy leaf margins. WOLF (**19**) expertly describes the symptoms which were also observed in this investigation: ". . . the internodes remain short, apical dominance is lost, and an unusually large number of leaves appear, oftentimes two to three hundred in a single plant. Such plants have the appearance of a rosette or witches' broom, and therefore the severe form of frenching may properly be called 'polyphylly'. . . ."

WOLF (**19**) has reviewed the reports and investigations of frenching up to 1935. Apparently the malady is of physiological origin since it has been impossible to isolate an organism or inoculate plants by using virus technique. Among the causes suggested, emphasis is placed upon field conditions, particularly a subsoil of hard-pan nature which prevents root penetration and also results in poor drainage of surface moisture. Frenching has also occurred as an aftermath of heavy rainfall. Investigations conducted with various forms of nitrogen and calcium compounds have given results so inconsistent that lack or excess of these elements forms no basis as a primary cause. Neither does soil reaction offer a good approach to an answer, although beneficial effects have been noted in soils with low pH values and when acids have been applied to soils producing frenched plants. The soil toxin theory has been investigated, but, here again, the experiments led to inconclusive results.

When portions of frenched tobacco plants, containing a growing point, were grafted on to healthy tobacco plants the later growth of the graft lost its frenching habit (**17, 18**). Such experiments not only indicate the noninfectiousness of the causal agent, but also eliminate the possibility of a genetic character being involved.

MCMURTREY (**5**) and SPENCER (**11**) have demonstrated that symptoms

[1] Published as Contribution No. 655 of the Massachusetts Agricultural Experiment Station.

and control of toxicity from thallium are similar to those of frenching. SPENCER and LAVIN (**13**) using spectrographic analytical technique did not find thallium in the tops of frenched plants grown in field soil. However, this lack of evidence in the tops of frenched plants did not eliminate thallium as a primary cause, since the action may have taken place in the roots, creating some disturbance which affected the tops. SPENCER (**12**) has demonstrated in both sand and water cultures that sulfanilamide and sulfapyridine, which also contains the sulfanilamide structure, produced a toxic condition in tobacco with leaf shapes similar to those produced when frenching is present. Closely related compounds failed to act in the same manner, which suggests, that frenching of tobacco may result from the production in the soil, or in the plant, of a toxic material with a structure similar to sulfanilamide. Bordering upon the toxin theory are the results of STEINBERG (**16**) that diffusates in agar of products from the activity of presumably nonpathogenic soil bacteria had a deleterious effect on tobacco seedlings expressed in symptoms of abnormality. STEINBERG (**14, 15**) obtained symptoms of frenching on tobacco seedlings in aseptic cultures with the amino acid *dl*-isoleucine. Other amino acids failed to induce frenching symptoms. Steinberg also suggests that mineral nutrition disturbances may affect protein metabolism and create excesses of amino acids and so lead to formation of charateristic deficiency symptoms.

Evidence now available points to some alterable soil constituent as a factor which disturbs the normal phytochemical balancing processes with the results that growth activity in the apical regions become abnormal and the characteristic shape of the tobacco leaf is changed and the phenomenon is observed as frenching.

Methods

The several experiments with soil have been carried out by utilizing 2-gallon containers which fit into a constant soil-temperature apparatus (1). The seedling tobacco plants were first established in a healthy growing condition at a soil temperature of 21° C. No attempt was made to maintain a definite air temperature other than to set the thermoregulator so that the air temperature would not fall below 21° C. The changing of the soil temperature from low to high, or vice cersa, was a matter of about 24 hours. All procedures were kept as simple and as uniform as possible in order not to burden the investigation with complications and uncertainties. Since it was established that the frenching would develop at a soil temperature of 35° C. and that normal growth was always obtained at a soil temperature of 21° C. these two temperatures constitute the high and low values unless otherwise specified. The experiments have been repeated several times for the purpose of checking on various features.

The total length of the apparatus is about 25 feet made up of 13 separate tanks. Light conditions differ slightly at either end of the apparatus, but it

was definitely determined it was only soil temperature that was involved in the production of frenched plants. The variety of tobacco, *Nicotiana Tabacum* L., used in the experiments was Havana Seed, a variety commonly grown in the Connecticut Valley for binder material in cigars.

The soil moisture was replenished with water from the tanks in which the containers were set, a procedure which did not alter the soil temperature. These tanks were lined with either galvanized iron or copper. The containers were galvanized iron. Thus there arose the question of either zinc or copper toxicity. It was, however, established that the nature of the material in the tanks or containers was not involved in the pattern of frenching.

For solution culture experiments it was possible to use the same tanks and their control mechanisms by inserting a temporary shelf to hold the quart preserve jars. Owing to danger of capsizing due to lightening of the jar by loss of water from transpiration it was not advisable to have the tank water level more than half the height of the jar. Temperature readings of the nutrient solution showed that this procedure was satisfactory in maintaining the desired temperature. The jars were in duplicate, one set having plants and the other set reserved for renewing the solutions by filling the jars and placing this reserved set in the tank for at least four hours to adjust the temperature of the solutions before the cultures were shifted. In this way fluctuating temperature were avoided.

Experimental results

Frenching masks mosaic symptoms

Tobacco was grown in a range of soil temperatures from 10° C. to 35° C. (50° F. to 95° F. with intervals of 5° F.). Half of the plants at each temperature were inoculated with a mosiac virus. Within a period of 12 days the mosaic symptoms were apparent on all inoculated plants. Gradually frenching symptoms developed at the soil temperatures of 32.5° C. and 35° C. on both the inoculated and uninoculated plants. The nature of the frenching symptoms was such as to obscure the mosaic symptoms (fig. 1). That the frenching did not destroy the virus was demonstrated by inoculating healthy plants with the juice of a frenched leaf from a plant previously inoculated with the virus. Keeping the plant at an ordinary greenhouse temperature, characteristic mosaic symptoms were again obtained, but the frenching features were absent. Healthy plants were similarly inoculated with the juice of a frenched leaf from a plant that had not been inoculated with the virus. Neither mosaic nor frenching symptoms became evident which proved that, since mosaic symptoms did not appear, the original uninoculated plant had not become contaminated by the virus. And, since frenching symptoms did not appear when healthy plants were inoculated with the juice of frenched leaves, it follows that the cause of frenching is not transmissible by the ordinary inoculating technique.

This experiment was supplemented by altering the soil temperatures by a complete reversal of the extremes of the range; i.e., the soil originally at 10° C. was raised to 35° C., and that which had been at 35° C. was lowered to 10° C. Since the high soil temperature of 35° C. had stopped terminal growth and the shift from 10° C. to 35° C. stopped terminal growth, the growth that developed after the shift came from the shoot development of axillary buds. The virus-inoculated plant developed a strong axillary shoot at the new soil temperature of 10° C., the succeeding

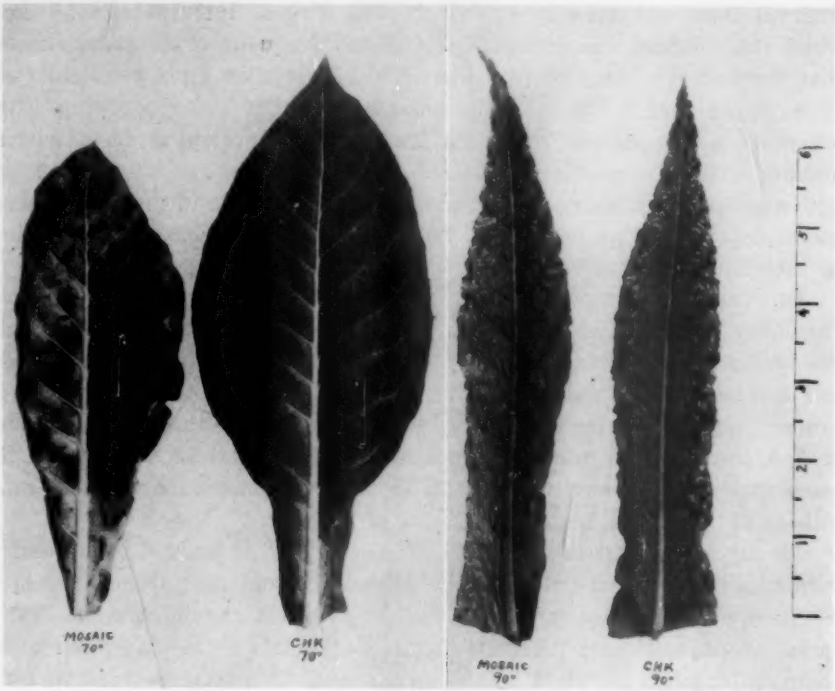

FIG. 1. Tobacco leaves from plants inoculated and uninoculated with a mosaic virus and grown at the two soil temperatures of 70° F. and 90° F. At right mosaic is masked by frenching.

leaves became more and more normal in shape and the common characteristics of the mosaic disease again became apparent. On the other hand, the plants which had been at the low soil temperature of 10° C. and were later subjected to a soil temperature of 35° C. developed frenching symptoms which completely masked any symptoms of mosaic in the inoculated plants. The uninoculated plants at this new temperature of 35° C. also developed equally good frenching symptoms.

These experiments demonstrated that the particular frenching obtained at the high soil temperatures was independent of the mosaic virus; that it did mask the symptoms of the mosaic virus but did not destroy its virulence.

Soil temperature effects on frenching

Exploratory experiments demonstrated that frenching would occur in plants at a soil temperature of 35° C. and that it never occurred when the soil temperature was 21° C. For convenience in obtaining a sufficient number of replications these two temperatures were preferred to a range of temperatures. As experience in observation of frenching increases, the symptoms are apparent earlier, and such information regarding the length of time to induce frenching must be considered as a longer time interval than was actually the case. The longest interval was 58 days while the shortest was eight. Careful and frequent observation showed that the first symptoms of frenching could be seen as a pin-head chlorosis of a young leaf. This usually became apparent on a morning after observation had shown that such leaves were developing in a normal manner with a normal green color. The disease became more apparent with a greater degree of chlorosis in succeeding leaves, and these succeeding leaves developed with less width, narrowing to a strap-leaf type and then by intermittent stages to the filform shape.

In general, a compost soil was used, since this soil always produced frenching, When two soils from tobacco fields were run parallel with the compost soil the frenching symptoms developed later than in the compost soil and of the two field soils one was 38 days later than the other in producing the frenching symptoms. Respectively, these three soils, compost, field A, and field B, induced frenching in 11, 20, and 58 days under the same experimental environment at 35° C. soil temperature. The same soils at 21° C. failed to show any signs of frenching.

In any of the experiments thus far performed, it has not been possible to distinguish a clear-cut division between normal and frenched leaves. There appear a series of gradations which gives the impression that a series of physiological reactions takes place during the development or complete elimination of frenching symptoms. While these reactions are slowly taking place new leaves are forming and developing with increasing or decreasing symptoms of frenching. Such a series of lessening symptoms was obtained by shifting to 10° C. a soil which had been at 35° C. The plant was well frenched at the top which had lost dominance so far as shoot elongation was concerned. This can be observed in fig. 2 which shows the frenching of leaves obtained at 35° C. (no. 1). At 10° C. the axillary shoot (nos. 2–3) developed the leaves being quite frenched at the base of the shoot (no. 2) and becoming less frenched as time and distance increased to point no. 3.

On the other hand a plant grown at the soil temperature of 10° C. had normal wide leaves (no. 1 in fig. 3). At 35° C. the plant lost dominance in its growing tip where a rosette of frenched leaves, (no. 2) developed. Axillary shoots bore leaves which were decidedly frenched (no. 3).

There was one other way by which the effect of soil temperature

expressed itself by altering the shape of the leaves. Two sets of plants growing in soil respectively at 21° C. and 35° C. were cut back, the soil nitrogen renewed by an application of fish meal, and the soil temperatures reversed. So-called sucker leaves developed and 11 days later these leaves

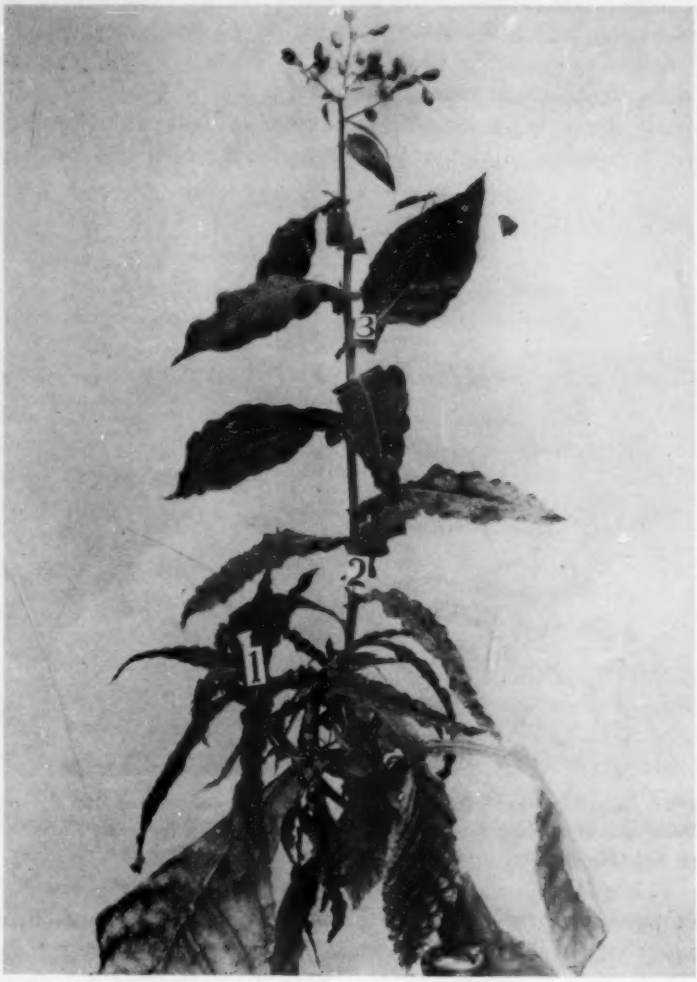

FIG. 2. Tobacco plant grown, first at a soil temperature of 35° C. and then at 10° C. Note progressive widening of leaves between points 2–3.

had all the characteristics that had been associated with the soil temperatures before they were reversed. Three days later, i.e., 14 days after shifting, the new or reversed temperatures began to express themselves. The sucker leaves which had started with signs of frenching were so immature that the new temperature of 21° C. was able to express itself by blunting of the apical ends as if an effort was being made to assume a normal width. The

basal ends continued to remain narrow, giving the impression that, because of the maturity of these cells, the tissues were fixed and the morphology in this region could not be altered. The suckers eventually elongated to form axillary shoots and at the end of the experimental period, 65 days, of the shifted temperatures the lower leaves, which were formed under the influence of the high soil temperature of 35° C. were frenched, while the upper leaves formed under the influence of the low soil temperature of 21° C. were, to all appearances, quite normal.

Likewise, the same time lag of 14 days was evident in the development of frenching symptoms on sucker leaves of plants which had been grown at

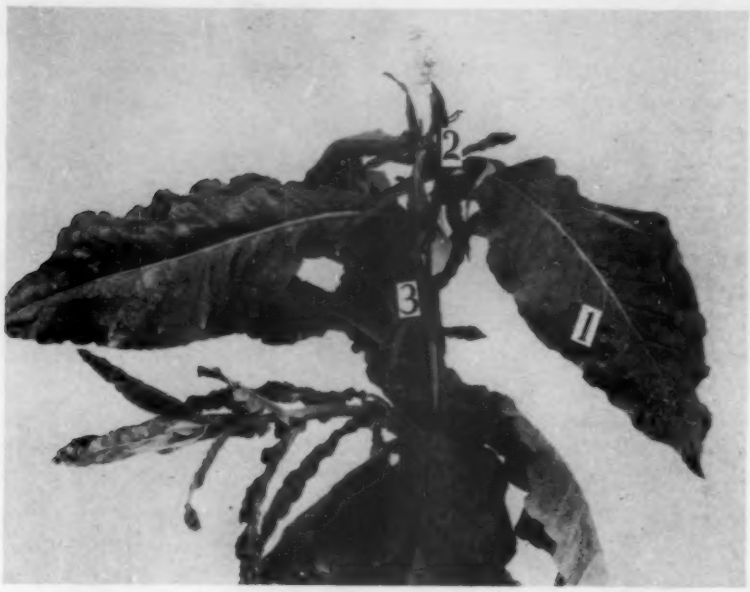

FIG. 3. Tobacco plant grown, first at a soil temperature of 10° C. and then at 35° C. Note wide leaf at 1 from low soil temperature and frenched leaves at 2 and 3 from high soil temperature.

21° C., topped, and shifted to the higher soil temperature of 35° C. The first leaves that developed were normal, but after the 14-day interval there appeared the first symptoms of frenching, i.e., pin-head chlorosis on the newer leaves. As time went on and these axillary shoots elongated the lower leaves remain normal, but the symptoms of frenching became accentuated in succeeding leaves beyond those that first showed the pinhead chlorosis.

These experiments demonstrate that there is a pattern of the physiological disease of frenching of tobacco related to soil temperature. In general, a soil temperature of 35° C. will induce frenching, but a soil temperature of 21° C. will not induce it. This latter temperature will also

cause the failure of frenching symptoms, on a frenched plant, to appear on leaves developed after the soil temperature has been altered from the high of 35° C. to 21° C. This general statement should be qqualified with the knowledge that the appearance of symptoms, as well as the failure of symptoms to appear, is subject to a time lag. That this pattern can be altered by air-drying of soil will be reported in another section of this paper. This pattern was not altered by the addition or withholding of applications of nitrogen, although there were cases where the intensity of the symptoms was mitigated when nitrogen was applied. Since most of the experiments were carried out with a compost soil which is a mixture of good top soil with animal manure and consequently of a high level of fertility there was little gain to be expected from the addition of nitrogen which was proved when such applications were made.

A discussion of the nitrogen factor as well as some other factors that altered the pattern of the onset of the disease will be considered in another part of this paper. The point involved is that soil temperature can play an important role in inducing the disease. However, temperature is not a substance that can enter into the composition of matter as a tangible constituent. Its function is of a regulatory nature. Any demonstration that results in altering the pattern to any considerable degree should give a basis for understanding what factor, or factors, is involved in the regulating effects of temperature and lead more directly to the true cause of frenching.

Varietal susceptibility to frenching

Although Wolf (19) recognizes that "all varieties of cultivated tobacco are susceptible to frenching" it was deemed desirable to check the variety used in these experiments (Havana Seed, a cigar tobacco), against a variety not commonly grown in the Connecticut Valley. Plants of the variety, Yellow Mammoth, a cigarette tobacco, were grown from seed obtained from Georgia. Both varieties were grown at the two soil temperatures of 35° C. and 21° C. The first frenching symptoms, pinhead chlorosis and recurved leaves, were observed on both varieties in eight days at the higher soil temperature. Thirteen days later terminal growth of the main stalks had apparently stopped and rosetting at the tip was well established with many small leaves appearing at the same time. Fig. 4 shows the condition of the plants 34 days after the experiment was started. It was interesting to note that the symptoms of frenching developed within the same time interval on both varieties and were equally intensified as the time period increased. No frenching symptoms were observed on either variety grown at the lower soil temperature of 21° C. It would appear that any frenching symptoms observed on the variety Havana Seed used as an indicator plant in these experiments will probably hold for other varieties as the Havana Seed is not a variety more susceptible to frenching than Yellow Mammoth.

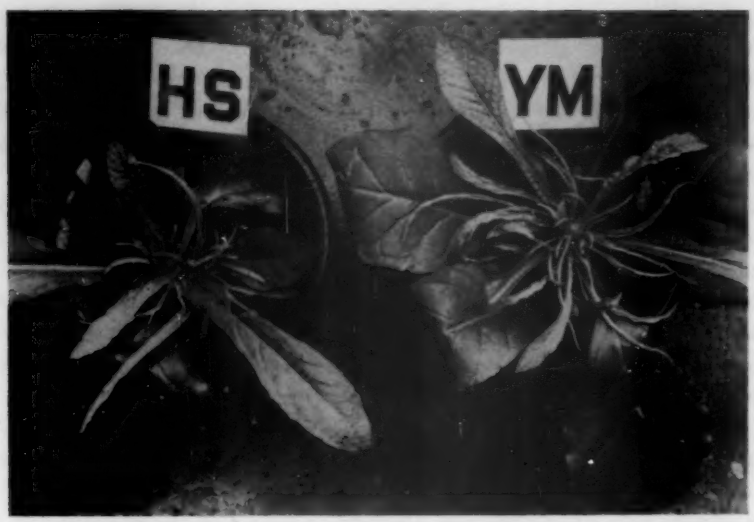

FIG. 4. Showing frenching symptoms on two varieties of tobacco. Havana Seed (HS) and Yellow Mammoth (YM).

SOLUTION CULTURE FOR FRENCHING SYMPTOMS

The almost unfailing procedure of obtaining frenching by a soil temperature of 35° C. and the experimental results indicating that excesses or deficiencies of mineral elements were not involved prompted the suggestion that temperature might exert its influence on the metabolic processes taking place in the roots. If soil temperature could be narrowed down to root temperature the problem of the cause of frenching might also be narrowed. Consequently tobacco plants were grown by solution culture technique, using SHIVE's R_5S_2 (9) as a basic formula for a nutrient solution. The same two temperatures of 35° C. and 21° C. were used and obtained by placing a platform in the water bath. The results were negative.

Even the introduction of soil extracts from soils containing frenched plants failed to produce frenching of plants growing in a nutrient solution with a solution temperature of 35° C. The extracts were made by shaking the frenching soil with about twice its volume of distilled water and passing the extract through filter paper. SHEAR (8) obtained frenching of tobacco grown in sand cultures by using the leachings from a soil that produced frenching. WOLF (19) tried the same experiment with three soils known to produce frenching and although he failed to obtain frenching with the leachates, he did obtain frenching on the check plants on which he used distilled water. SHEAR's (8) results prompted him to conclude that frenching is a toxicity disease and not a deficiency disease.

Solution culture tests were made by exploring several possibilities. The NH_4-ion was introduced by ammonium sulfate and various proportions of the NH_4-ion and the NO_3-ion (from calcium nitrate) were tested. The NH_3-

ion from ammonium hydroxide was tried in the absence of the NO_3-ion, the calcium being obtained from calcium chloride. The nitrite radical from sodium nitrite was tested in the absence of the NO_3-ion. Iron from ferrous and ferric compounds failed to induce symptoms of frenching in solution culture. The only evidence of symptoms of frenching was obtained by leaving iron out of the culture solution. At the end of the experimental period of 44 days the minus iron cultures showed narrower leaves, a slight general chlorosis, wavy margins, greater turgidity, and a slight stunting.

Effect of zinc, copper, calcium, and boron

The tanks for maintaining constant soil temperatures were lined with either copper or galvanized iron and the containers in which the plants were grown were of galvanized iron. This suggested that, at the higher temperature, 35° C., copper or zinc ions might have some influence on frenching. Copper or zinc ions could come from the tank water which, because it was at the same temperature as the soil in the cultures, was used to replenish the water supply of the soil in which the plants were growing. Zinc ions were common to all the containers since they would be in contact with the soil from the galvanized iron of the containers.

Calcium and boron ions were considered along with copper and zinc, more as an exploratory experiment than with the thought that they are important in the frenching of tobacco. Wolf (**19**) observed severe frenching of tobacco where limestone had been used on a field. Plants grown on the strip where the limestone was applied had previously always been normal, and the areas adjacent to the limed and frenched strip grew normal tobacco plants.

Some of the symptoms of boron deficiency are not so unlike symptoms of frenching as to be entirely excluded from consideration. McMurtrey (**4**) noted the yellow color and leaf distortion when there is a deficiency of boron. Lack of boron prevented the setting of seed pods, but in this investigation failure to set seed pods was not apparent with frenching induced by soil temperature. To isolate the soil from contact with galvanized iron, thereby eliminating contact with zinc, one-gallon glazed crocks were set into the two-gallon galvanized containers and surrounded with water. This amounted to a double water bath, the temperature of the smaller one controlled by the larger. Ordinary tap water was used for replenishing soil moisture, but this was heated to the required soil temperature before it was applied.

The exclusion of copper and zinc ions from the soil did not alter the pattern of the onset of frenching, for frenching appeared on plants at the high soil temperature of 35° C. and did not appear at the lower soil temperature of 21° C. By reversing the soil temperatures the new growth on the frenched plants developed without frenching characteristics and the nonfrenched plants became frenched when the soil temperature was raised from 21° C. to 35° C. When calcium hydroxide was added to the soil the plants showed

benefit in the form of better growth, but in no way was there an alteration in the pattern of temperature-induced frenching.

The results with boron were slightly different, borax being the source of the B-ion. The application was a little too heavy as some of the plants were killed shortly after being transplanted into the soil. Replacements usually lived, but frequently the leaves had burned margins and interveinal areas. Frenching later appeared according to the pattern of soil temperature influence. Delay in appearance of frenching symptoms may have been due to the slow growth of the plants caused by toxicity from too much borax, or the borax may have affected some biological activity in the soil. Reversing the soil temperatures resulted in the elimination of symptoms on new growth and the appearance of symptoms on the original nonfrenched plants.

Soil reaction relationships

Wolf (19) gives an adequate review of the literature concerning the observations and experiments with the frenching of tobacco and hydrogen-ion concentration of the soil. In general, the evidence shows that as the pH values of the soil approach the neutral point of 7.0 frenching symptoms are commonly found earlier than on more acid soils. The acidification of the soils by acids delayed frenching but an application of sulfur to a soil one year in advance of setting the plants seemed to have eliminated frenching on this soil while the plants in the adjacent area developed frenching symptoms.

Although soil reaction may possibly be altered by high temperatures, there was no evidence that frenching induced by soil temperature was traceable to a soil reaction relationship. A compost soil which produced frenching in eight days at a soil temperature of 35° C. was sampled from two containers in two water baths. One soil had a pH value of 5.1, the other 4.9. Similar samples were taken from check containers at 21° C.—a soil temperature that does not produce frenching—and these had pH values of 5.1 and 4.95.

In another case in which two field soils were tested parallel with the compost soil there appeared no differences in pH values of the same soil at the different temperatures, but there were differences between soil.

Soil	Days to french	pH value
Compost	17	5.6
Field (A)	28	4.6
Field (B)	58	4.45

Though frenching is apparently delayed by a greater hydrogen-ion concentration in the soil, the fact remains that its appearance was associated with a high soil temperature.

Soil moisture relationships

The growing of plants in nonporous containers was attended with the problem of maintaining suitable soil moisture. Not only did soil tempera-

ture play a significant role in loss of water, but the size of the plant became an important consideration. The vagaries of New England weather, even with greenhouse culture, do not allow advance planning for water replenishment. Since it was not convenient to remove the containers for the purpose of adding water by weighing, the best system was to add water daily on the basis of judging what would be sufficient for a 24-hour period. When the plants were large the water was frequently added twice daily since the whole amount in one application might cause injury by saturating the soil.

There were occasions when the soil seemed relatively dry, although the plants were seldom observed to be in a wilted condition. Such dry soils were always at the high soil temperature of 35° C. This is the temperature that induces frenching symptoms. Several times following the drying of the soil new growth exhibited less severe symptoms of frenching. Such plants could always be thrown back to more intense symptoms of frenching by more frequent and copious watering.

The drying of the soil as a preventive of frenching was tested. A fresh sample of field soil was obtained, divided into two portions, one of which was kept moist and the other air dried. This field soil was used parallel with a compost soil which was known to produce frenching at the high soil temperature of 35° C. In this experiment the compost soil produced frenching in 20 days and the moistened field soil in 24 days, but the air-dried portion of field soil did not produce frenching within the experimental period of 67 days. All soils were at the temperature of 35° C.

WOLF (**19**) made a similar observation concerning the inability of three sandy soils to produce frenching after storage in a greenhouse for a few months. On the other hand, he reports that two clayey soils did not lose the frenching quality after a year in storage. He further noted that SCHWEIZER (**7**) dried frenching soils in the sunlight thereby nearly eliminating frenching symptoms when later tested. Schweizer interpreted these results as indicative of the presence of a toxin as the cause of frenching.

NITROGEN RELATIONSHIPS

VALLEAU and JOHNSON (**17**) made an exhaustive study with various inorganic and organic nitrogenous substances as a cure for frenching. They had observed that frenching followed nitrogen deficiency symptoms, and for the most part, obtained complete recovery of plants by applying nitrogen after the onset of frenching. SPENCER (**10**) controlled frenching by repeated applications of a nitrogenous fertilizer. SHEAR (**8**) obtained recovery of frenching plants after adding nitrogen to the soil.

In these experiments, in which frenching has been induced by soil temperature, nitrogen, added to the soil, has not effected any cure nor prevented the appearance of frenching symptoms. Fish meal plus a little potassium nitrate has been the source of nitrogen. On some plants

it was noted that the quite yellow frenched leaves assumed a darker color after an application of nitrogen, but the new growth continued to show all the symptoms of frenching. Nitrogen was frequently added to the soil, since it was part of the experimental program to maintain a fairly high level of this nutrient. Leaf tissue tests for nitrates were made with diphenylamine (**2, 6**). These tests showed that the plants were not deficient in nitrogen. Check plants, purposely starved, gave negative tests for nitrates, thereby demonstrating that the chemicals and procedure were not at fault.

In one experiment one half the plants were allowed to exhaust the soil of nitrogen. The other half had the nitrogenous fertilizer added to the soil at two-week intervals. The added nitrogen had no effect on the height of the plants at the high soil temperature of 35° C. because the frenching interfered with growth. At the lower temperature of 21° C., where frenching was absent, the added nitrogen materially increased the size of the plants. Frequently the tests for nitrates showed up more strongly in the tissues of frenched plants. This indicated an ability of the plant to absorb nitrogen, but an inability to transform such nitrogen into protein materials.

Discussion

Although it has been possible to demonstrate with a compost soil that a relatively high temperature, 35° C., will consistently induce frenching of tobacco, no claim is made or implied that temperature is the primary cause. Whatever the primary cause may be, the results of this investigation strengthen the conclusions of other investigators who believe the primary cause to be a soil constituent. The fact that frenching may be induced by raising the soil temperature, and normal growth resumed by lowering the soil temperature, without the addition or subtraction of any soil constituent, other than water, strongly indicates that the ability of a soil to cause frenching is a latent quality which can and does become active under the impetus of exciting agents which are unrelated, but which produce a common end point, the primary cause of frenching.

Temperature is not a constituent. Its role can be only that of regulating physico-chemical reactions or influencing some biological activity of soil flora. This latter speculation is supported by the evidence that, even at a high soil temperature, plants develop growth less symptomatic of frenching in soil on the dry side than in soil with a high moisture content. Air drying of the soil also rids the soil of frenching potentialities. While it is possible to interpret this information as suggesting the presence of a toxin which might come from biological activity, it also suggests that such biological activity could cause a competition between soil flora and higher plants for certain nutritive requirements. Soil temperature could alter the balance of soil flora which, to a great extent, affects the rate of mineralization and the amount of elements available for nutrition of higher plants.

Summary

1. Tobacco plants were grown in a compost soil in a constant soil temperature apparatus. Frenching was induced at the high soil temperature of 35° C. and was absent at the soil temperature of 21° C. or lower. Frenching symptoms masked the symptoms of a mosaic virus, but did not destroy its virulence. It was not possible to transmit frenching symptoms by the ordinary technique of inoculation.

2. The first symptoms of frenching appear as a pinhead chlorosis and a narrowing of the developing leaf. In one case the symptoms appeared as early as eight days in the soil temperature apparatus and in another case as late as 58 days, induced by the high soil temperature of 35° C.

3. Altering the soil temperature from a high 35° C. to 21° C., or lower, caused a progressive lessening to complete disappearance of frenching symptoms on the subsequent growth of the plant.

4. There was a considerable time lag between the exposure to soil temperature and the appearance or lessening of the frenching symptoms.

5. The variety Yellow Mammoth (a cigarette tobacco) and the variety Havana Seed (a cigar tobacco) were equally susceptible to temperature-induced frenching.

6. Plants grown at a root temperature of 35° C. by solution culture technique failed to develop symptoms of frenching. Nutrient solutions into which filtered water extracts from frenching soils were introduced failed to show that the cause of frenching could be transmitted by this procedure.

7. Slight symptoms of frenching were observed when iron was left out of the nutrient solution.

8. The elimination of copper and zinc ions from the applied water did not alter the pattern of temperature-induced frenching.

9. Calcium hydroxide benefited the growth of tobacco plants but had no apparent influence on the onset of frenching.

10. Borax affected growth and delayed the onset of frenching but did not affect the pattern of the temperature influence.

11. There was no relationship between the pattern of frenching induced by soil temperature and soil pH values, but it was confirmed that lower pH values delayed the onset of frenching symptoms.

12. Air drying of a soil destroyed its ability to produce frenching symptoms. Growing the plants in a soil with a lowered average moisture content lessened the symptoms of frenching on new growth.

13. The addition of a nitrogenous fertilizer in the form of fish meal reinforced with potassium nitrate failed to prevent or cure frenching induced by a soil temperature of 35° C.

14. Plant tissue tests for the nitrate ion gave positive results on frenched plants. The qualitative tests were more intense with frenched plants than with nonfrenched plants, which indicated an ability on the

part of the frenched plant to absorb nitrogen, but an inability to transform all the nitrogen to protein materials.

The seed of the cigarette tobacco variety, Yellow Mammoth, was supplied by J. G. Gaines, Associate Pathologist, Division of Tobacco Investigations, U.S.D.A., B.P.I., at Tifton, Ga.

DEPARTMENT OF BOTANY
AGRICULTURAL EXPERIMENT STATION
UNIVERSITY OF MASSACHUSETTS, AMHERST

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UNAVAILABILITY OF IRON AS A CAUSE OF FRENCHING OF TOBACCO (*NICOTIANA TABACUM* L.)[1]

LINUS H. JONES AND MARCO A. TIO

(WITH TWO FIGURES)

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Introduction

In continuation of the work reported by JONES (**4**) the effort has been directed toward investigating the factors in the soil which induce frenching of tobacco (*Nicotiana Tabacum* L.) at a soil temperature of 35° C. and which are impotent at a soil temperature of 21° C. Previous work (**4**), while not discounting the soil toxin theory, did suggest that biological activity of soil flora might alter the availability of a nutrient element and thereby upset a physico-chemical balance which might disturb normal development of the plant. Since it is recognized that a soil flora is a complicated society affected by environment, particularly moisture and temperature, no attempt has yet been made to sort out the various organisms to ascertain which, if any, might be responsible for depriving a plant of its requirements.

During the process of the investigation certain experiments were performed and special studies made, which, although the results had no bearing on the final conclusions, are reported in this paper as contributions pertinent to information on the subject of frenching.

Methods

Tobacco plants, variety Havana Seed, were used throughout the experiments. The seed were germinated in a soil-sand mixture and later pricked out into flats of soil.

The soil temperature apparatus (**2**) was altered by constructing platforms in the separate tanks in order to support the 2-liter battery jars which were used as plant containers. Two soil temperatures were employed, a high of 35° C., which is known to induce frenching, and a low of 21° C. at which frenching does not occur. Replenishment of water was at the same temperature of the soil and was obtained from the water bath in which the containers were set. The soil was an ordinary greenhouse compost soil prepared for potted plants and with a higher level of fertility than a field soil.

Experimental results

RESULTS WITH SOIL DILUTIONS

A compost soil was diluted with sand as shown in table I. The object was to determine the effect of a lowered level of fertility on the rapidity of

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the onset of frenching. The test was conducted at a soil temperature of 35° C. with one set of duplicate check cultures of all sand and all soil at 21° C. To the sand cultures was added sufficient potassium nitrate solution, nitrogen equal to 70 p.p.m., to saturate the sand. Frenching occurred in all cultures at 35° C., in two cases as early as nine days, and all cultures at 35° C. had frenched in 24 days. No frenching symptoms were evident in the check cultures of all sand and all soil at 21° C. within the 45-day period of the experiment.

There was little evidence that a pattern could be made from the factors, time to french and levels of soil fertility as measured by soil dilution. If frenching is due to a mineral deficiency in the soil such a deficiency would have been aggravated by dilution with sand, and, in general, frenching would have appeared much earlier in the cultures with the greater amounts of sand. On the other hand, if frenching is due to a soil toxin the dilution

TABLE I

RELATIVE EFFECT OF VARIOUS PROPORTIONS OF SAND AND SOIL MIXTURES (BY VOLUME) AT 35° C. ON HEIGHT OF PLANTS, ONSET OF FRENCHING, AND HYDROGEN-ION CONCENTRATION (PH VALUES) OF SOIL

MIXTURE BY VOLUME		HEIGHT OF PLANTS 31 DAYS	ONSET OF FRENCHING	PH VALUES	
SAND	SOIL	CM.	DAYS	ORIGINAL	FINAL
9	0*	18.0	21	6.80	6.77
8	1	15.9	9	6.72	6.65
7	2	4.9	9	6.70	6.64
6	3	7.0	15	6.50	6.35
5	4	10.4	14	6.43	6.34
4	5	13.8	11	6.31	6.25
3	6	15.7	24	6.27	6.16
2	7	17.3	12	6.21	6.01
1	8	16.8	12	6.20	5.93
0	9	17.5	15	6.20	6.82

* Received enough KNO_3 (N = 70 p.p.m.) solution to saturate culture.

with sand would have diluted the toxin and frenching would have appeared earlier with the lesser amounts of sand. The results of the observations of the number of days that passed before frenching symptoms were evident does not follow either one of these hypotheses (table I).

It is known that the culture which was the last to develop frenching symptoms, (sand 3 parts—soil 6 parts), 24 days, became dry several times. The drying of a soil is known to delay, prevent, and cause the lessening of frenching symptoms on subsequent growth (**4**).

There was no relation between the height of the plants and days to french. In general, however, the greater heights were in the cultures less diluted with sand. In table I are also recorded original and final pH values of the cultures and here again there is no relationship to the time element for the appearance of frenching symptoms.

Effect of autoclaved soil

Shear (7) used several methods for partially sterilizing a frenching soil. Autoclaving and chloroforming soils killed weed seeds and destroyed the ability of the soil to produce frenching. A formaldehyde dust treatment of the soil did not kill weed seeds and did not destroy the frenching potentialities. Shear concludes that these results, considered in relation to his other tests, support a toxicity disease theory as opposed to the frequently expressed view that frenching is a deficiency disease. Valleau and Johnson (10) made use of an autoclave to heat a known frenching soil to between 65° and 75° C. Unheated soil produced frenching; the autoclaved soil did not. The addition of virgin soil, about 10 per cent., to a heated soil induced frenching. The preventive effect obtained from heat is ascribed to a greater availability of soil nitrogen.

Spencer (8) obtained similar results by adding small amounts of soil to a quartz sand. Sand alone did not produce frenching. A sand mixture containing 0.05 per cent. soil caused frenching within five weeks; a 1.0 per cent. soil within four weeks; and a 10.0 per cent. soil within three weeks. In another experiment Spencer (8) demonstrated that a washed quartz sand, without the addition of soil could produce frenching symptoms. Spencer concluded that a toxic material was present in the tap water obtained from a deep well and that this toxic material eventually became sufficiently concentrated from repeated watering. Schweizer, according to Wolf (11), partially sterilized soils by air-drying in the sunlight, causing them to lose their ability to induce frenching. He interpreted such results as indicative of a toxin as the cause of frenching. Jones (4) not only demonstrated that the air-drying of a soil caused it to lose its ability to produce frenching, but also observed that intense symptoms of frenching became less intense on subsequent growth following lessened water supply in the soil. Jones expressed less concern with a toxin theory, but stressed the possibility of a more active soil flora at a high soil temperature competing more successfully than tobacco for nutrient material.

In order to demonstrate the effect of a soil temperature influence on tobacco grown in autoclaved sand and soil and checked against nonautoclaved sand and soil the following experiments were conducted. Autoclaving was done at 15 lbs. pressure for one hour. To the sand cultures was added enough potassium nitrate solution (N = 70 p.p.m.) to saturate the soil. Even this was not sufficient to prevent a nitrogen deficiency chlorosis, since all sand cultures, even in the absence of frenching, were chlorotic. Even the nonautoclaved soil culture at 35° C. was chlorotic, but the autoclaved soil culture at 35° C. was not chlorotic. It is possible that the autoclaving of the soil made sufficient nitrogen available for the needs of the plants within the time limit of the experiment, 45 days. It will be noted in table II that frenching occurred in the cultures of nonautoclaved sand and soil at 35° but no frenching occurred in the cultures of autoclaved sand and soil. The frenching symptoms appeared on the plants in the non-

TABLE II

EFFECT OF AUTOCLAVING SAND AND SOIL ON THE DEVELOPMENT OF FRENCHING AND CHLOROSIS AT SOIL TEMPERATURES OF 35 AND 21° C.

SOIL TEMPERATURE	CULTURE	TREATMENT	FRENCHING	CHLOROSIS
°C	No.			
35	1	Autoclaved sand	Minus	Minus
	2	Autoclaved soil	Minus	Plus
	3	Plain sand	Plus	Minus
	4	Plain soil	Plus	Plus
21	5	Autoclaved sand	Minus	Plus
	6	Autoclaved soil	Minus	Plus
	7	Plain sand	Minus	Minus
	8	Plain soil	Minus	Plus

autoclaved soil in 15 days and in 19 days on plants in the nonautoclaved sand (at 35° C.). At 21° C., a soil temperature that evidently does not induce frenching, no frenching symptoms were apparent. Figure 1 is a photograph of these cultures showing the relative amounts of growth.

This experiment was repeated under slightly modified conditions. The sand was inoculated with one part (by volume) of a soil, which was at the time producing frenching, to eight parts of sand. No nitrate was added to the sand cultures. This inoculated sand and undiluted soil were autoclaved and planted with tobacco seedlings. Similar cultures of nonautoclaved inoculated sand and soil, as checks, were also established. The two soil temperatures of 35° C. and 21° C. were again employed. Frenching occurred only at the 35° C. soil temperature and only on plants in the inoculated sand and the soil which had not been autoclaved (table III).

This type of experiment was again modified by confining the test to soil alone and mixing the soil with autoclaved soil and mixing it with nonautoclaved soil. The tests were performed at the soil temperatures of 35° C. and 21° C. (table IV). Frenching occurred within 16 days, only at the high soil temperature and only from the soils that contained nonautoclaved material. There was no evidence at all that autoclaving produced any compound that destroyed a possible toxin in so-called frenching soils. This

FIG. 1. Tobacco plants at high and low soil temperatures. At left, 35° C. 1 steamed sand, 2 steamed soil, 3 plain sand, 4 plain soil. At right, 21° C. 5 steamed sand, 6 steamed soil, 7 plain sand, 8 plain soil.

TABLE III

EFFECT OF AUTOCLAVING INOCULATED SAND AND SOIL ON THE PRODUCTION OF FRENCHING AND THE IRON CONTENT OF PLANTS GROWN AT SOIL TEMPERATURES OF 35° C. AND 21° C.

Soil temperature °C.	Growing Medium		Autoclaved	Frenching	Iron content of plants
	Parts				
	Sand	Soil			Fe p.p.m.
35	8*	1	.....	Plus	33.01
	0	9	.....	Plus	36.00
	8*	1	Plus	Minus	130.20
	0	9	Plus	Minus	111.30
21	8*	1	.....	Minus	92.76
	0	9	.....	Minus	96.70
	8*	1	Plus	Minus	91.50
	0	9	Plus	Minus	92.25

* Sand inoculated with a frenching soil.

experiment was repeated three times and consistently showed that two liters of autoclaved soil inoculated with a small handful of nonautoclaved soil would carry the frenching factor. On the other hand, at no time did an uncontaminated autoclaved soil produce frenching. However, such frenching symptoms as did appear were produced at the high soil temperature of 35° C.

If a toxin theory is to be substantiated a soil with frenching potentialities might be expected to give evidence of the toxin at 21° C. But this is not in evidence. The results of these experiments confirm the results of JONES (**4**) to the effect that, with comparable cultures and with soil temperature the only variable, frenching occurs at the soil temperature of 35° C. In these experiments frenching has never occurred at the soil temperature of 21° C., although good growth of the plants takes place at this lower temperature. If a toxin is naturally present in the soil, or if its source is from the water used for replenishment of soil moisture there seems to be no justification for supplementing the toxin theory with the qualification that the toxin is active only at the high soil temperature. It is possible to conceive that such an imaginary toxin is latent and only awaits activation to exert its influ-

TABLE IV

EFFECT ON FRENCHING ABILITY OF A MIXTURE OF AUTOCLAVED AND NONAUTOCLAVED SOILS

Treatment	Frenching Soil temp.	
	21° C.	35° C.
No treatment	Minus	Plus
Inoculated with autoclaved soil	Minus	Plus
Autoclaved, inoculated with nonautoclaved soil	Minus	Plus
Autoclaved	Minus	Minus

ence. Also, one can conceive that this imaginary toxin is generated by microbial activity, neutralized under normal conditions, but not neutralized effectively under conditions of a high soil temperature. Even this conception has its limitations for, in general, the higher the organic content of the soil, such as compost, the less will be the extent of injury from detrimental influences originating in the soil. In all these experiments it has been generally observed that earlier responses to frenching influences are obtained from compost soils than from field soils.

The results seem to justify, even though they do not prove, a theory that soil organisms were involved and, at the high soil temperature of 35° C., were sufficiently active to compete more successfully than the tobacco plant for some element or elements required for normal growth. The early symptoms of frenching, pinhead chlorosis, suggested a plant deficiency of either nitrogen or iron. Since frenching had been induced from soils with a relatively high level of fertility, periodically enriched with nitrogen, and the plants showed positive presence of the NO_3-ion in plant tissue (**4**), the direction of the investigation was toward iron relationship, although other elements were not neglected.

Response to iron salts

Since it has been definitely determined that frenching can be prevented by dropping the soil temperature to 21° C., or by use of accepted means for partially sterilizing the soil by heat or chemicals, it can be safely concluded that the cause of frenching is not a deficiency of a nutritive element in the soil itself. On the other hand, if it could be ascertained that the plant itself was lacking in some required element there would be a basis for a more direct attack upon this problem of frenching. The discovery of such a theoretical deficiency would not be the complete answer to the problem concerning the primary cause of frenching, but it should narrow the field of investigation with the hope that a positive approach is possible.

Working on the theory that a required nutritive element, although present in the soil, was lacking in the plant tissue, a means was sought to introduce such elements into affected leaves. The means decided upon was a modification of that used by Phillis and Mason (**6**) who floated discs of cotton foliage on a sugar solution in order to study the formation of starch. Various very dilute solutions of salts of the several major and minor mineral elements (table V) were substituted for the sugar solution. Petri dishes with 50 ml. of solution were employed to culture the foliage discs. The discs were cut with a frequently sharpened 20 mm. cork borer.

Twelve discs for each solution were cut from frenched leaves and an equal number from normal foliage of healthy plants as checks. Discs of frenched and normal leaves were floated on distilled water as controls. The Petri dishes, with their covers on were placed in the greenhouse with cheesecloth laid over the dishes to prevent excessive light. After 48 hours there was definite recovery from the chlorosis typical of frenching in discs floated

TABLE V

MAJOR AND MINOR MINERAL ELEMENTS IN SOLUTION FOR TESTING DEFICIENCIES IN FLOATING DISCS FROM FRENCHED LEAVES

ELEMENT	SALT	MOL. SOLN.	DILUTION	CONCENTRATION	RECOVERY INDUCED
		gm. per l.	*ml. per l.*	*p.p.m.*	
Magnesium	$MgSO_4 \cdot 7H_2O$	246.50	2.00	5	–
Manganese	$MnSO_4 \cdot 4H_2O$	223.05	0.05	3	–
Iron	$FeSO_4 \cdot 7H_2O$	278.01	0.10	6	+
Zinc	$ZnSO_4 \cdot 6H_2O$	269.54	0.02	1	–
Aluminum	$Al_2(SO_4)_3$	342.12	0.05	1	–
Boron	H_3BO_3	61.84	0.05	0.5	–
Copper	$CuSO_4 \cdot 5H_2O$	249.71	0.02	1	–
Phosphorous	KH_2PO_4	136.13	1.00	32	–
Potassium	KNO_3	101.10	2.00	78	–
Nitrogen	NH_4NO_3	80.50	5.00	140	–

on the ferrous sulfate solution. Ten of the 12 discs showed a deep green color and no yellow mottling was observed in any of them. The discs from normal leaves did not show changes in color. These tests were repeated and other tests tried, modifying the disc method by using whole leaves partially immersed in the solutions. However, the disc method gave the most consistent results.

Since the iron sulfate solution was the only one that initiated partial or complete recovery of the chlorosis in frenched leaves, tests with discs were made to learn the effect of concentration and also the possibility of substituting the ferric ion (from ferric phosphate) for the ferrous ion (table VI). Within the 48-hour period of the test there was definite recovery of all discs floating on the ferrous sulfate solutions. However, at 15 p.p.m. of Fe and above there was iron toxicity from the ferrous ion which was visible as a dark brown perimeter and a few brown spots within. There were recovery symptoms when the iron was from ferric phosphate, but they were no more intense at 30 p.p.m. than at 10 p.p.m. The response to the

TABLE VI

SHOWING EFFECT OF CONCENTRATION OF IRON FROM FERROUS SULFATE AND FERRIC PHOSPHATE UPON THE RECOVERY AND TOXICITY TO 12 DISCS FROM FRENCHED LEAVES

CONCENTATION OF IRON	FERROUS SULFATE		FERRIC PHOSPHATE	
	RECOVERY	TOXICITY	RECOVERY	TOXICITY
p.p.m.	*No. of discs*	*No. of discs*	*No. of discs*	*No. of discs*
0	0	0	0	0
5	12	0	1	0
10	12	0	3	0
15	12	10	3	0
20	12	12	3	0
25	—	—	5	0
30	—	—	3	0

ferrous iron was, therefore, encouraging, but it appeared doubtful that any great reliance could be placed upon ferric iron as a basis for preventing or correcting frenching symptoms.

The form in which iron appears in the soil available for absorption by plants is not definitely known. A search of the literature gave no tangible evidence whether such iron was inorganic or organic in nature or what physical, chemical, or biological reactions made soil iron soluble and available to plants. There is plenty of evidence that chlorosis of plants can be induced by a deficiency of iron brought about by heavy applications of lime. This is associated with a low hydrogen-ion concentration of the soil solution. Iron, supposedly, reacts to form various complex compounds with soil constituents and exists in a so-called "fixed" condition. It probably does not disappear from the soil in drainage waters though there is considerable evidence to show it may be leached to some extent and takes part in the formation of hardpan. In general iron is seldom thought of as a possible deficient element in soils. Usually the plants obtain sufficient iron from soils to meet their nutritive requirements. Since the iron is absorbed through the root system it must exist in a soluble form even though its concentration in p.p.m. is very low.

For purposes of experimentation the authors felt that the ordinary salts of iron applied to the soil would be quickly rendered unavailable if used in small amounts or would be toxic if used in large amounts. They assumed that a mixture of an iron salt with peat would give an iron humate compound with a lower degree of ionization and a lessened toxic power than would be obtained from a direct application of an inorganic salt of iron. Two mixtures were prepared using both ferrous sulfate and ferric phosphate. Equal weights of these salts were mixed with equal weights of a dry sedge-reed peat. To each mixture was added five times its weight in water. After thorough mixing, the material was allowed to react and dry for two weeks. At the end of this period the material existed as a dry cake which was finely ground before using.

These mixtures were applied to a diluted soil (8 parts sand to 1 part soil) in battery jars and set to three tobacco plants. The plants were grown at the known frenching soil temperature of 35° C. with two control cultures to which no iron was added, one of the controls being undiluted soil.

A preliminary test was made in which the three materials, peat, ferrous sulfate, and ferric phosphate, were used separately. The results indicated that these iron salts were not as effective by themselves in preventing frenching as they were when mixed with peat, and that peat was not as effective by itself as it was when mixed with an iron salt, particularly ferrous sulfate. Consequently the further effort for tracing the primary cause of frenching was devoted to testing the iron and moisture content of samples of foliage taken from the plants subjected to the study of the effects of the peat mixtures with the two iron salts.

The data in table VII give an outline of this experiment with such per-

TABLE VII

EFFECT OF FERROUS AND FERRIC IRON MIXTURES WITH PEAT ON HYDROGEN-ION CONCENTRATION, TOXICITY TO PLANTS, FRENCHING, GENERAL CONDITION OF PLANTS, AND IRON CONTENT OF PLANTS AFTER 35 DAYS AT 35° C. SOIL TEMPERATURE

	Controls		$FeSO_4$-peat				$FePO_4$-peat			
Soil dilution { Sand / Soil	8 / 1	0 / 9	8 / 1				8 / 1			
Iron added by volume	*None*	*None*	*0.01%*	*0.02%*	*0.04%*	*0.08%*	*0.01%*	*0.02%*	*0.04%*	*0.08%*
pH values { Initial	6.43	6.60	6.43	6.43	6.43	6.43	6.43	6.43	6.43	6.43
pH values { Final	5.98	6.49	6.36	6.25	5.71	5.48	6.56	6.62	6.65	7.25
Toxicity symptoms	–	–	–	–	+	+	–	–	–	–
Frenching symptoms	+	+	–	–	–	–	+	+	+	–
Condition of plants	Poor	Poor	Healthy	Healthy	Healthy	Healthy	Chlorotic	Chlorotic	Chlorotic	Chlorotic
Iron p.p.m.	33.01	36.00	348.70	423.20	379.20	437.90	22.36	35.50	26.30	129.64

tinent information as is applicable to the immediate discussion. Frenching appeared in all cultures except those containing the ferrous sulfate-peat and the highest amount of ferric phosphate-peat. Some toxic symptoms were apparent in the two highest ferrous sulfate-peat cultures. In table VII are also recorded the initial and final pH values of the separate cultures. The ferrous sulfate-peat had a definite tendency to increase the hydrogen-ion concentration of the soil, due probably to the SO_4 radical. Where ferric phosphate-peat was used the hydrogen-ion concentration of the soil decreased, and it is believed this was due to the increase in buffering by the PO_4 radical. It will be noted that there were no frenching symptoms on plants grown with the highest amount of ferric phosphate-peat, yet this culture had a pH value above the neutral point of 7.0. It will also be noted that the iron content, 129.64 p.p.m. in the foliage of this culture was considerably higher than the iron content of the other three cultures supplied with ferric phosphate-peat. The next highest ferric phosphate-peat culture had but 26.3 p.p.m. There is a closer association of results between iron content and lack of frenching, than between pH values and frenching. Practically no benefit was obtained from the peat itself since this would have been apparent with the ferric phosphate-peat mixture had peat included this property of correcting or alleviating frenching.

The iron content of the above-ground parts of the plants was determined. At the conclusion of the experiment with the growing plants, 35 days, the plants were cut off at the ground level, weighed green, dried in an oven at 60° C. for 48 hours, dry weight determined and ground (table IX). The iron in terms of parts per million of dry weight was obtained by the spectrophotometric method of FORTUNE and MELLON (1).

It will be observed in table VII that wherever frenching occurred there was a relatively small amount of iron in the plants. All these cultures were at 35° C. This table (table VII) is supplemented by iron data in table IV which shows that at 21° C. the autoclaved diluted and undiluted soils had approximately the same iron content in the plants, but at 35° C. there was between three and four times as much iron in the plants from autoclaved soils as from unautoclaved soils. It becomes apparent that in those cultures where frenching occurred there was a relatively low content of iron in the plants. In those cultures to which the ferrous iron was supplied in a peat mixture there was a high content of iron in the plants and no frenching. Where ferric iron was supplied in a peat mixture there was frenching and a low iron content in the foliage except in the culture receiving the largest application, which prevented frenching and gave a higher content of iron in the plants than was found in any of the nonfrenching cultures in table III where the soil temperature was 21° C.

One may conclude, therefore, that autoclaving a soil destroys or limits some factor, probably biological, normally active at 35° C. and not so active at 21° C., which materially decreases the iron normally available to the tobacco plant and that this lack of iron affects the morphological development of the plant to bring about abnormally shaped leaves.

That other nutrient requirements are not involved may be explained on the basis that the diluted soil (8 parts sand—1 part soil) gave practically the same iron content (table III) at 21° C. as whole soil and practically the same iron content whether the soils were or were not autoclaved. Had other nutrients been involved it would follow that the addition of supplemental iron (table VII) would not have so greatly altered the iron content or affected the ability of the soil to cause frenching. These experimental results conclusively confirm the tests with floating discs on various solutions of separate nutrients, from which it was evident that the only response of the chlorotic discs of frenched leaves was from an iron solution.

Moisture and osmotic relations

In cases of the extreme evidence of frenching, such as the strap and filiform leaves, the turgidity of the foliage is very marked. Frequently such leaves will split from a margin to a midvein. That frenched leaves do have a higher percentage of moisture has been reported by Wolf (11). The following data confirm this earlier work. The information was obtained from frenched plants grown at 35° C., from normal plants grown at 21° C., and from apparently normal plants which had the benefit of supplemental iron grown at 35° C.

TABLE VIII

Relation of green weight, dry weight, and percentage of moisture in frenched and normal leaves of plants grown at soil temperatures of 35° C. and 21° C., respectively

Sample	Frenched leaves 35° C.			Normal leaves 21° C.		
	Green weight	Dry weight	Moisture	Green weight	Dry weight	Moisture
No.	*gm.*	*gm.*	*%*	*gm.*	*gm.*	*%*
1	6.0550	1.0011	83.5	4.7641	1.4051	70.5
2	5.0940	0.7931	84.4	4.0483	1.2369	69.5
3	7.8420	1.2099	84.5	4.4482	1.2518	72.0
4	7.4250	1.2251	83.5	3.9081	1.1983	69.5
5	8.6871	1.2537	85.5	4.3882	1.3108	69.5
Avg.	.....	.....	*84.28*	.....	.....	*70.50*

Table VIII gives the green weight, dry weight and percentage of moisture of frenched and normal leaves. Since there was a wide difference in soil temperature, some cultures at 21° C., others at 35° C., one might raise the question regarding the influence of soil temperature on the moisture content of plants. The answer is found in the results in both tables VIII and IX. In the latter table (table IX) the data are recorded from cultures, all of which were grown at the higher soil temperature of 35° C. It will be noted in table IX that frenching occurred in cultures to which no iron had been added and in the three lowest applications of the ferric phosphate-peat mixture. Frenching was prevented by the application of all amounts of

TABLE IX

EFFECT OF IRON-PEAT MIXTURES ON FRENCHING AND THE GREEN WEIGHT, DRY WEIGHT, AND PERCENTAGE OF MOISTURE OF LEAF SAMPLES ON PLANTS GROWN AT A SOIL TEMPERATURE OF 35° C.

	Controls		$FeSO_4$-peat				$FePO_4$-peat			
Soil dilution										
Sand	8	0	8				8			
Soil	1	9	1				1			
Iron added by volume	*None*	*None*	*0.01%*	*0.02%*	*0.04%*	*0.08%*	*0.01%*	*0.02%*	*0.04%*	*0.08%*
Frenching	*Plus*	*Plus*	Minus	Minus	Minus	Minus	Plus	Plus	Plus	Minus
Samples										
Green weight, *gm.*	2.847	2.913	2.067	1.753	2.449	1.895	4.489	3.729	4.937	2.243
Dry weight, *gm.*	0.4556	0.4662	0.5374	0.5086	0.7591	0.5496	0.6734	0.5967	0.6912	0.5833
Moisture, %	84	83	74	71	69	71	85	84	86	74

the ferrous sulfate-peat mixture and by the highest application of the ferric phosphate-peat mixture. The data on percentage of moisture is fairly consistent in that the figures are lower in the samples from the normal plants and this checks with the figures in table VIII on samples from normal plants grown at the low soil temperature of 21° C. The difference in percentage of moisture is therefore not due to soil temperature itself. It is linked with an iron availability in the soil which was altered by soil temperature relationships in a natural soil, but unaffected by soil temperature when the soil was autoclaved.

The osmotic concentration of normal and frenched leaves was determined by both the plasmolytic and cryoscopic methods. For the plasmolytic method a series of sucrose solutions was prepared varying by increasing the concentration by 0.05 M from 0.1 M to 0.8 M. Sections of frenched and

TABLE X

OSMOTIC CONCENTRATION IN ATMOSPHERES OF THE CELLS OF FRENCHED AND NORMAL LEAVES DETERMINED BY THE PLASMOLYTIC METHOD WITH SUCROSE

CONCENTRATION OF SUCROSE SOLUTION	EQUIVALENT OSMOTIC PRESSURE	FRENCH LEAVES					NORMAL LEAVES				
		SAMPLE NOS.					SAMPLE NOS.				
M	*atm.*	*1*	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*	*5*
0.1	2.6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.15	4.0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.20	5.3	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.25	6.7	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.30	8.1	.....	.....	.....	.....	.....	Plus	.....	Plus	.....	.....
0.35	9.6	.....	.....	.....	.....	.....	.....	Plus	.....	Plus	Plus
0.40	11.1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.45	12.7	.....	Plus	Plus	.....	.....	.....	.....	.....	.....	.....
0.50	14.3	Plus	.....	.....	Plus	Plus	.....	.....	.....	.....	.....
0.55	16.0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
0.60	17.8	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

normal leaves with a thickness of 15μ were cut with a microtome and dipped at once into the various sugar solutions. Incipient plasmolysis took place in the frenched tissues in the sugar concentrations of 0.45 M and 0.50 M and in the normal tissue at 0.30 M and 0.35 M (table X). These figures were compared with the table of URSPRUNG and BLUM (9) for the equivalents of osmotic pressures in atmospheres and show that the frenched leaves had a greater osmotic concentration than the normal leaves.

The cryoscopic method depends upon the depression of the freezing point by using a Beckmann thermometer. Frenched and normal leaves were ground in a clean porcelain mortar and the cell sap collected in test tubes which were immersed in a freezing mixture of ice and sodium chloride in a vacuum bottle. The depression of the freezing point was calculated by comparison with the freezing point of distilled water. The osmotic concentration of the cell sap in terms of atmospheres was determined from the formula.

$$p = \frac{\Delta \times 22.4}{1.86}$$

where Δ is the true depression obtained after determining the undercooling and correcting the apparent freezing point lowering for the undercooling error by means of the equation $\Delta = d - \frac{du}{80}$, where d is the observed depression and u is the undercooling. The results of this method are shown in table XI which shows the true depression and the corresponding osmotic concentration as determined by the formula. These results check with those of the plasmolytic method in that the frenched leaves have a greater osmotic concentration than the normal leaves and are in fairly close agreement. However, these results are in disagreement with those published by WOLF (**11**) who reports that normal leaves have a greater osmotic concentration than frenched leaves. The samples used by Wolf were from field soils which were probably at a much lower level of soil fertility than the compost soil used in the experiments reported in this paper.

HISTOLOGICAL EFFECTS OF FRENCHING

The effect of frenching is expressed in the color and the gross morphology of the leaves. In connection with the intensity of chlorosis there appears to be a direct relationship to the narrowness of the leaf. It would be expected that such radical shapes of frenched leaves would have histological characteristics different from normal leaves. WOLF (**11**) exhibits a drawing by Brecher showing the cross section of a string-leaf type in which the tissues of the blade show practically no differentiation from the rudimentary tissue. Photomicrographs of cross sections of both frenched and normal leaf blades are shown (fig. 2). The material was imbedded in paraffin according to the method described by JOHANSEN (**3**) and sectioned to a thickness of 10 μ. The sections were stained with Haidenhain's haematoxylin with orange G as a counterstain, and also with safranine with fast green as a counterstain.

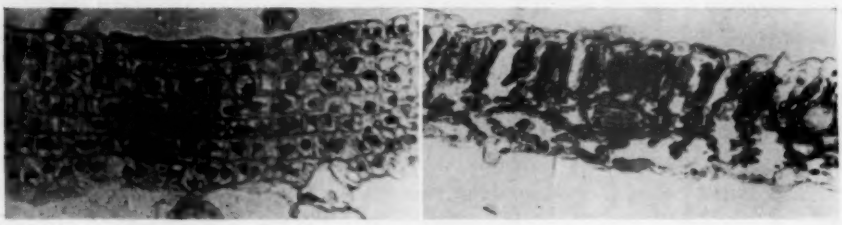

FIG. 2. Photomicrograph showing the difference in structural organization of the cells of the leaves of frenched and normal leaves. Left, frenched. Right, normal.

The sections show that in the frenched leaves there was not the definite organization of tissues that is present in normal leaves, i.e., epidermis, palisade layer, and spongy parenchyma. Since this investigation demonstrates that the abnormal leaves are a result of iron deficiency, the histologi-

TABLE XI

OSMOTIC CONCENTRATION OF THE CELLS OF FRENCHED AND NORMAL LEAVES DETERMINED BY THE CRYOSCOPIC METHOD

Sample	April 22, 1947				May 2, 1947*			
	FRENCHED LEAVES		NORMAL LEAVES		FRENCHED LEAVES		NORMAL LEAVES	
	True depression △	Osmotic concentration	True depression △	Osmotic concentration	True depression △	Osmotic concentration	True depression △	Osmotic concentration
No.	*°C.*	*atm.*	*°C.*	*atm.*	*°C.*	*atm.*	*°C.*	*atm.*
1	1.116	13.44	0.840	10.11	1.052	12.67	0.711	8.56
2	1.106	13.31	0.701	8.44	0.998	12.02	0.701	8.44
3	1.099	13.23	0.742	8.93	1.067	12.85	0.691	8.32
4	0.993	11.95	0.717	8.63	1.115	13.42	0.726	8.74
5	0.986	11.87	0.696	8.38	1.039	12.51	0.685	8.25

* The leaves, frenched and normal, tested on May 2, 1947, were younger and less advanced in frenching symptoms than those used April 22, 1947.

cal condition suggests that iron in the plant may play a role beyond that of simply being necessary for the formation of chlorophyll.

Discussion

The evidence supports a theory that a biochemical reaction is the initiating factor that results in an iron deficiency in the tobacco plant; this would be the primary cause of frenching. A maintenance of soil temperature somewhere near 35° C. seems to activate some soil organism which utilizes iron in performing some vital function, thereby competing for soluble iron more successfully than the higher plant, tobacco in this instance. This particular fraction of soil flora is, apparently, less active in utilizing soluble iron at a soil temperature of 21° C., thus affording the tobacco plant an opportunity of satisfying its iron requirement at this temperature.

The organisms involved are eliminated, or considerably suppressed, by processes of partial sterilization of the soil and their activity or numbers materially reduced by a low soil moisture content, i.e., soil on the dry side. This checks with the observations of WOLF (**11**) who reports that frenching in the field was associated with heavy rainfalls shortly prior to the onset of frenching. He noted further that poorly drained areas were observed where frenching was present and that small local areas lacking frenched plants were well drained. This relationship, as well as a temperature relationship, is involved. Both of these factors have a recognized marked bearing on microbiological activity and the intensity of such biochemical effects.

The tobacco plant and its closely related genera may not be the only group of plants that suffers from unavailable soil iron due to the effect of warm soils. The senior author was recently asked to diagnose the cause of lack of growth coupled with a mild chlorotic condition of the foliage of rose plants. The time was August, the weather hot, and the greenhouse hotter. A manure mulch covered the ground beds in which the plants were "sick." Obviously a lack of nitrogen was not the cause of lack of vigor. The floating disc test showed a deficiency of iron in the foliage. Rapid and complete recovery followed an application of a peat and ferrous sulfate mixture. At the same time roses in other houses on this same range were not affected by the warm weather. The soil in these houses had been allowed to air-dry in order to make the roses dormant for the purpose of cutting back. The plants had since been rejuvenated by watering and were quite vigorous in their new development. It is quite possible, as with air-dry soils and frenching of tobacco, that the drying of the soil about the rose plants destroyed that theoretical fraction of soil flora which otherwise would have made soil iron unavailable to the plants.

The theory of a biochemical reaction at a high soil temperature as an agent in making iron unavailable to higher plants requires definite proof that such organisms do exist and that their introduction from pure culture into partially sterilized soils will produce the characteristic symptoms of frenching. A complete knowledge of these organisms in environment of

both high and low soil temperature may offer the explanation as to why the various theories as to the cause of frenching have been left in such a divergent and confused state. Further experimentation is necessary to establish practical recommendations for the prevention and cure of iron deficiencies in plants that are grown in soils which frequently reach relatively high temperatures. The ferrous sulfate mixture with peat offers a good basis for continued investigation, as both materials are fairly abundant and relatively inexpensive. Ferrous sulfate is the copperas of commerce and peat is quite generally available.

This investigation does demonstrate that the symptoms of frenching may be prevented if sufficient available iron is present in the soil. It is well known that an iron deficiency can induce chlorosis. That an iron deficiency may alter the morphology of a leaf is not generally known. In the case of frenching soils such symptoms of narrow leaves were prevented when iron was available through the application of peat mixtures, but the symptoms were evident in the checks or when too small quantities of the ferric phosphate-peat mixture were employed. In this connection it is interesting to note that OWEN (**5**) reports a narrowing of the carnation leaf when iron was withheld from a nutrient solution in sand culture.

Summary

1. Tobacco plants were grown in battery jars of about 2-liter capacity at soil temperatures of 21° and 35° C. A compost soil with a high level of fertility was used in whole or diluted with sand. Tests were made on the ability of these soils to cause frenching with whole soil, diluted soil, autoclaved soils, autoclaved soils inoculated with fresh soil, and the preventive effects of ferrous and ferric iron mixtures with peat.

2. Studies on mineral deficiencies by the floating disc method were made. Determinations of moisture content, iron content, and osmotic concentration of the cell sap were made with frenched and normal leaves.

3. The dilution of soil with sand did not greatly affect the ability of such dilutions to cause frenching at the soil temperature of 35° C. No frenching occurred on plants grown in whole sand or whole soil at 21° C. In this test there was no correlation between pH values and frenching.

4. Frenching was prevented by autoclaving sand and soil when growing the plants at a soil temperature of 35° C., checked against unautoclaved sand and soil at the same soil temperature of 35° C. which did produce frenching. However, unautoclaved sand and soil at 21° C. failed to produce frenching.

5. When autoclaved soil was inoculated with a small amount of unautoclaved soil, the ability to cause frenching was returned to the soil, but no deleterious effects to frenching ability occurred when nonautoclaved soil was inoculated with autoclaved soil. It was only at the higher soil temperature of 35° C. that frenching occurred. These results justify a theory that the soil flora, at a soil temperature of 35° C., is functioning in competi-

tion with higher plants for a certain required element or elements requisite for the normal growth of the tobacco plant.

6. Tests made on plant tissue of frenched plants by the floating disc method gave response only to an iron salt solution.

7. When peat mixtures of ferrous sulfate and ferric phosphate were mixed with a frenching soil and plants exposed to a soil temperature of 35° C., frenching was prevented in all cultures to which the ferrous iron had been added and in the cultures to which the greatest amount of the ferric iron was added.

8. Chemical analysis of frenched and normal leaves showed a marked difference in their content of iron. Normal leaves from plants grown at a soil temperature of 21° C. had nearly three times as much iron in their leaves as leaves from corresponding cultures grown at a soil temperature of 35° C. The analysis showed also that where frenching was prevented by using the peat mixtures of ferrous sulfate and ferric phosphate there was a much larger content of iron in the leaves.

9. The moisture content of frenched leaves is higher than that of normal leaves, and the osmotic concentration of the cell sap, by both the plasmolytic and cryoscopic methods, is higher in frenched leaves.

10. Histological studies of frenched and normal leaves showed a marked lack of tissue differentiation in the frenched leaves.

11. The various experiments indicate that a high soil temperature of 35° C. encourages certain microbiological activity in the soil which disturbs the normal soluble iron content of the soil and results in an iron deficiency in the tobacco plant. The results in no way support a soil toxin theory nor do they afford evidence that any deficiency of a mineral element exists in the soil. It seems to be a question of unavailability of iron in the soil and a consequent deficiency in the plant.

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DEPARTMENT OF BOTANY
AGRICULTURAL EXPERIMENT STATION
UNIVERSITY OF MASSACHUSETTS, AMHERST

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PHOTOSYNTHESIS IN RELATION TO STOMATAL FREQUENCY AND DISTRIBUTION

R. O. Freeland

(WITH ONE FIGURE)

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Introduction

The concept that stomates play an all-important role in controlling the diffusion of carbon dioxide into leaves during photosynthesis seems to be quite generally accepted by teachers and authors of botany texts. In a plant physiology text by Maximov (**6**) may be found the following, "That the carbon dioxide enters the leaf mainly through the stomates may be shown by a simple experiment. If on a certain portion of the leaf the stomata are coated with vaseline, and the leaf is then exposed to light and afterward treated with iodine, the blue color reaction will be observed only in those portions where the stomata remained open." Also in the text by Meyer and Anderson (**7**) one finds "Critical experiments have shown, however, that the proportion of this gas (CO_2) entering the leaves by this route (directly through epidermis) is relatively small, and that practically all of the carbon dioxide entering leaves diffuses in through the stomates." Similar statements may be found in many other botanical books.

It seems that this concept stems mainly from the research of Blackman and his contemporaries. Blackman (**1**) in a review of the research regarding this problem concludes that "Under normal conditions, practically the sole pathway for carbon dioxide into or out of the leaf is by the stomata." Further support and impetus were given to this idea by the classical experiments of Brown and Escombe (**2**) which demonstrated and partially explained the enormous diffusive capacity of small pores and stomates. More recently Maskell (**5**) working with *Prunus lauro-cerasus* and Nutman (**9**) studying *Coffea arabica* have reported that photosynthesis in these plants is directly related to stomatal movement.

On the other hand considerable evidence has appeared in the literature which indicates that the rate of diffusion of carbon dioxide through epidermal cells of leaves may be quite appreciable and should not be minimized or ignored. Blackman mentions that Boussigault and Barthelmy reported data to the effect that the carbon dioxide exchange during "assimilation" was independent of stomatal distribution. Mitchell (**8**) found that the leaves of tomato and Pelargonium absorbed carbon dioxide and accumulated carbohydrates in appreciable quantities although the stomates appeared to be closed. Furthermore, the amount of carbon dioxide absorbed by the leaves in which the stomates appeared to be closed was approximately equal to the amount absorbed by the same leaves when

the stomates were open. HEINICKE and CHILDERS (4) and SCHNEIDER and CHILDERS (10) in their extensive research with apple trees, found many occasions when the rate of photosynthesis was quite high even though the stomates were closed. In this laboratory, many measurements of the apparent photosynthesis from the upper and lower surfaces of leaves of various plants have been made. As often as not the results have shown little or no direct correlation between photosynthesis and stomatal distribution. Some of these data will be presented in this paper.

Experimental procedure

The plants to be included in this report are yellow-green coleus, *Coleus blumei;* avocado, *Persea americana;* poinsettia, *Euphorbia pulcherrima;*

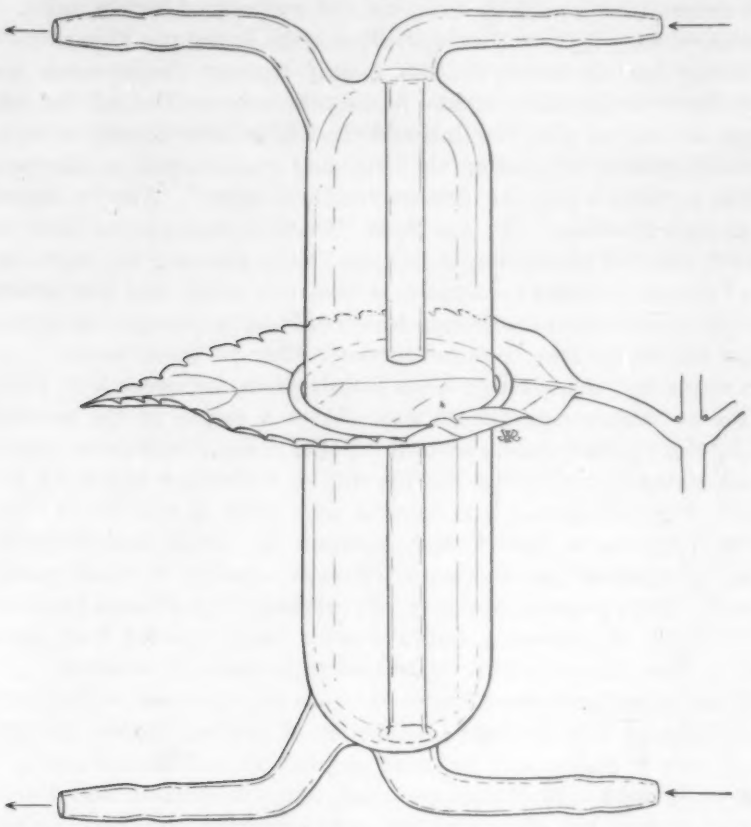

FIG. 1. Glass cups clamped on the top and bottom of a leaf. Description in text.

rubber plant, *Ficus elastica;* begonia, *Begonia sp.?; Rhoeo discolor;* bean, *Phaseolus vulgaris* (var. stringless green pod) ; tobacco, *Nicotiana tabacum,* and geranium, *Pelargonium zonale.* These plants were selected because of variation in stomatal distribution and thickness of cutiele. Six of the species have stomates in the lower epidermis while the other three have

stomates in both the upper and lower surfaces of their leaves as shown in table I. The amount or thickness of cuticle progresses from very little in such plants as coleus and tobacco to much in poinsettia and rubber plant.

Apparent photosynthesis, in terms of milligrams of carbon dioxide used, was measured from the upper and lower surfaces of the leaves. Glass cups of the design shown in figure 1 were clamped opposite each other on several leaves of the experimental plant. These cups each had a diameter of 4 cm. and a depth of 8 cm. A little grafting wax applied around the rim of each cup and warmed slightly at the time the cup was applied to the leaf provided an air-tight seal. Natural air passed through these cups to absorption towers containing 0.1N KOH for carbon dioxide determination after the method of HEINICKE and CHILDERS (**4**). Using flowmeters and constant suction, FREELAND (**3**), the rates of air flow were so regulated as to prevent excessive depletion of the carbon dioxide of the air and extreme temperature rise in the cups. A rate of air flow of about 0.5 cu. ft. per hour was adequate. Experimental plants were placed near a laboratory window in direct sunlight where on hot days they were shaded with one or two layers of cheesecloth. No other attempt was made to control the temperature in the cups or around the plants. Temperatures were recorded several times during the course of each experiment using thermocouples and a Leeds and Northrup thermopotentiometer. Little or no difference in temperature was ever found in the cups attached to the opposite sides of a leaf. The duration of each experiment was four hours.

To test the accuracy of the apparatus and methods, a large number of determinations of the carbon dioxide content of the air in the laboratory have been made. From these measurements it has been found that the milligrams of carbon dioxide per cubic foot of air can be determined with a standard deviation of 0.2.

Results and discussion

The experimental results obtained for the various plants are summarized in table I. The data presented for each plant were obtained by averaging the results from three or more determinations made upon different days.

An examination of the data for those plants which have stomates only in the lower epidermis provides a basis for evaluating the importance of stomates in the absorption of carbon dioxide during photosynthesis. For example, in coleus, avocado, and begonia, carbon dioxide diffused through the upper epidermis in very significant amounts. In no case was the rate of diffusion of carbon dioxide through the upper epidermis alone as great as it was through the lower epidermis plus stomates. However, from the known structure of leaves, it would appear to be a reasonable assumption that carbon dioxide could diffuse through the lower epidermal cells, excluding stomates, at a rate approximately equal to that through the upper epidermis. On this basis the apparent photosynthesis calculated for the

total leaf epidermis, excluding stomates, would really be twice the amounts presented for the upper epidermis in table I. Applying this principle to coleus, as an example, the apparent photosynthesis in mg. of CO_2 used per hour per square decimeter would be 6.2 through epidermal cells alone and 7.0 through stomates alone. The data for the other plants in this group, poinsettia, rubber plant, and *Rhoeo discolor*, indicate that they belong in a separate class. In these plants there is little or no diffusion of carbon dioxide through the epidermal cells and the stomates are the primary pathway through which carbon dioxide diffuses during photosynthesis. A partial explanation for the difference between these two groups of plants with respect to carbon dioxide exchange through the astomatous epidermis may be found in the thickness of the epidermis or cuticle. There probably are other factors as indicated by the data for begonia, which has a rather thick cuticle. At any rate the data for these plants, having stomates in the

TABLE I

SUMMARY OF THE DETERMINATIONS OF APPARENT PHOTOSYNTHESIS IN TERMS OF THE AMOUNT OF CARBON DIOXIDE WHICH DIFFUSED INTO THE LEAF THROUGH THE UPPER AND LOWER EPIDERMIS

PLANT	MG. OF CO_2/DM.^{2}/HR.		STOMATES/CM.^{2}	
	UPPER	LOWER	UPPER	LOWER
COLEUS	3.1	10.1	0	11,000
AVOCADO	4.1	5.9	0	15,200
POINSETTIA	0.6	4.1	0	13,000
RUBBER PLANT	*	2.3	0	17,000
BEGONIA	2.2	3.3	0	3,400
RHOEO DISCOLOR	*	3.5	0	1,700
BEAN	*	3.8	1,000	7,000
TOBACCO	3.1	3.6	3,000	3,000
GERANIUM	1.3	3.7	2,300	15,000

* Not significant.

lower surface of the leaves, indicate that the statement of Blackman and others that the stomates are practically the sole pathway for carbon dioxide into and out of leaves under normal conditions is not true in many cases. The data confirm the conclusions of Mitchell, Heinicke, and others to the effect that some plants may carry on a rather high rate of photosynthesis with the stomates closed.

The results for bean, tobacco, and geranium which have stomates in both the upper and lower epidermis of the leaves must be examined from a different point of view. For these plants the amounts of apparent photosynthesis related to stomatal diffusion and epidermal diffusion respectively cannot be separated. In tobacco there is a direct correlation between stomatal frequency and apparent photosynthesis for the upper and lower side of the leaves. As indicated above there is no way of determining how much, if any, of the carbon dioxide exchange was due to epidermal diffusion exclusive of stomates. For geranium the degree of correlation is

much less and for bean it is probably nonexistent. Therefore, one must conclude from these data that for those plants having stomates in both the upper and lower epidermis of their leaves the diffusion of carbon dioxide into the leaves during photosynthesis may or may not show a direct correlation with stomatal frequency and distribution. It seems probable that in some plants, at least, some other factor (or factors) other than stomatal frequency and distribution plays a considerable role in determining the pathway of carbon dioxide exchange during photosynthesis.

Summary

1. This is a report of measurements made to determine the relative significance of stomates versus the epidermis, exclusive of stomates, as routes followed by carbon dioxide which diffuses into leaves during photosynthesis.

2. Plants were selected for the experiments to obtain wide variations in stomatal frequency and distribution, and thickness of cuticle.

3. For those plants having stomates only on one side of their leaves, considerable variation occurred with respect to the absorption of carbon dioxide through the epidermal cells during photosynthesis. In all cases the rate of apparent photosynthesis through the stomatal bearing epidermis was greater than through the astomatous side. In some plants the amount of carbon dioxide exchange through epidermal cells alone was very significant sometimes being approximately equal to the amount which diffused through the stomates. In other plants of this group, particularly those with a thick cuticle, little or no apparent photosynthesis could be detected through the epidermis without stomates.

4. The data for those plants having stomates in both the upper and lower epidermis indicate that apparent photosynthesis, in terms of CO_2 absorption, may or may not show a direct correlation with stomatal frequency and distribution.

DEPARTMENT OF BOTANY
NORTHWESTERN UNIVERSITY
EVANSTON, ILLINOIS

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GASEOUS LOSSES OF NITROGEN FROM GREEN PLANTS. II. STUDIES WITH EXCISED LEAVES IN NUTRIENT MEDIA

F. E. Allison and Luann De Tar Sterling

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In the previous paper (**1**) of this series, studies to determine whether gaseous losses of nitrogen occur from cultures of Chlorella and Lemna when grown on various sources of nitrogen, showed that no losses occurred except for one minor exception. In this one case slight losses of nitrogen from Chlorella cultures, grown on ammonium nitrate, did occur. The significance of this finding is somewhat in doubt, however, since these cultures were nearly dead when analyzed, due to increasing acidity during growth. The work, of course, with this exception, failed to corroborate the findings of Pearsall and Billimoria (**9**).

Most of the work of Pearsall and Billimoria (**9, 10**) was with sterilized Narcissus leaves floated on nutrient solutions. This technique provides conditions that are far from normal for growth, but it offers one of the few practical means of obtaining quantitative data on nitrogen losses resulting from the metabolism of common flowering plants. Where inorganic sources of nitrogen were present, losses of more than 50 per cent. of the total nitrogen added were sometimes observed by these workers. The indications were that nitrogen was lost by interaction of nitrites with amino nitrogen, both being products of plant metabolic processes. Under conditions where protein formation was proceeding, the nitrogen losses were small. Where protein hydrolysis predominated the rate of nitrogen, loss was often large. Similar experiments conducted by Mothes (**8**), using leaves of *Phaseolus multiflorus*, Hippeastrum, and Agapanthus, failed to confirm these findings.

In view of the obvious practical and theoretical importance (**5, 12**) of these observations of Pearsall and Billimoria, further work of the same type was initiated. In these experiments, which are reported below, the floating leaf technique similar to that used by Pearsall and Billimoria was adopted, but the analytical procedures were very different.

Experimental methods

The test plants used included the leaves of Iris, Belamcanda, and Narcissus. In all cases fully developed, healthy leaves were chosen. They were cut off near the bulb and used as soon as possible. The sterilizing solution was calcium hypochlorite made up according to the method of Wilson (**13**). Ten grams of the calcium hypochlorite was added to 140 ml. of water and this solution was then diluted 10-fold.

Considerable difficulty was experienced in finding a satisfactory procedure for handling and sterilizing the leaves. It was, of course, not only

necessary to sterilize them with the minimum injury, but also to reduce the experimental error in the nitrogen determinations to the minimum. The general procedure followed was to weigh together lots of two to four leaves (cut or whole), varying with different experiments, and place them in 500-ml. Erlenmeyer flasks for sterilization. Approximately 400 ml. of the calcium hypochlorite solution was added to each flask. These were stoppered and put on a shaker for 10 minutes to make certain that the entire surface of the leaves was constantly wet during the sterilization period. Various concentrations of calcium hypochlorite, times of treatment, and methods of manipulation were tried before adopting the procedure described. The time of sterilization chosen did not always give complete sterilization but was usually effective and produced a minimum of leaf injury.

In the preliminary work the unsterilized leaves were cut longitudinally into halves, one half being used for the determination of nitrogen originally present in the leaf, and the other half being incubated after sterilization. Each half was also cut transversely into suitable lengths for use in one-liter culture flasks. By this method the weights of each half could be made directly, but the cut edges were badly bleached during sterilization and undoubtedly the normal metabolism of many of the plant cells was destroyed. The data reported below in table I were obtained with leaves thus sterilized after cutting.

In the second procedure, used in obtaining all of the remaining data reported below, the leaves were sterilized before cutting. The manipulations needed for whole leaves, which were the usual aseptic procedures using sterile gloves, scissors, and forceps, were obviously more difficult with respect to preventing contamination after sterilization. Only the control half could be weighed conveniently, and the incubated half was calculated by subtraction of the control half from the whole. Evaporation from the control leaves before weighing was checked by placing them in a saturated atmosphere for the short time that elapsed between cutting and weighing. After sterilization, the leaves were slit longitudinally, and half of each leaf was cut up and placed in the culture medium. In this procedure most of the flasks showed no contamination after 60, or more, hours of incubation, unless the sterilization itself had not been adequate.

The culture medium on which the leaves were floated was the same as that used by Pearsall and Billimoria. Its composition in grams per liter was KH_2PO_4, 8.7168; K_2HPO_4, 0.4648; and glucose 30. The rate of addition of NH_4NO_3 (or the nitrogen equivalent of KNO_3 or NH_4Cl) varied and is shown in the tables. The pH of this medium was 5.1 to 5.3. Portions of it, usually 100 ml., were added to one-liter flasks; and, after plugging, were sterilized at 15 pounds pressure for 10 minutes.

The leaves, partly immersed and partly floating on the nutrient medium, were incubated at 23° C. under a bank of fluorescent lights for a period of 60, or more, hours. Any cultures that showed bacterial or fungal growths

were usually discarded, although in some cases, noted later, analyses were made to determine whether the microörganisms had affected the nitrogen content.

In preparation for analysis the control halves of the leaves were placed in a desiccator containing P_2O_5 at room temperature and evacuated to the vapour pressure of water. Fresh P_2O_5 was added and the desiccator again evacuated as often as necessary. After about 48 hours the plant samples were completely dry and ready for Kjeldahl analysis. This method of drying was chosen in preference to heat because of less chance for nitrogen losses. The incubated leaves, after decanting off the culture solution, were washed two or three times with distilled water and dried by the same procedure used for the control halves. The medium and leaf washings were made up to a volume of 250 ml. and aliquots taken for analysis.

Total nitrogen including nitrates, was determined on the dried leaves by the usual salicylic acid modification of the Kjeldahl method, and on the medium by the Davisson-Parsons method. Attempts were made to analyze the wet leaves and the combined leaf and medium samples by the latter method, but the results were unsatisfactory. Correction values, expressed in milligrams of nitrogen and based upon the slight differences between the weights of the approximate leaf halves, are given in the tables that follow.

The experimental procedures and methods described above could not, for obvious reasons, be expected to lead to results of extreme accuracy. The necessity for working with green plant material that lost moisture during weighing, and where the original nitrogen content of a plant sample could not be determined directly, were biological sources of error. The chemical errors were those always inherent in the Kjeldahl methods. In the present studies three such nitrogen determinations entered into each final loss or gain figure. These included the analyses of the control half leaf, the incubated portion, and the medium. Usually only a fifth of the medium was taken for analysis and hence any slight error is magnified five-fold in arriving at the total nitrogen content. Although the methods had their limitations they were as good as could be devised without the benefit of the isotopic technique.

Experimental results

A large number of experiments to test for nitrogen losses using the floating leaf technique were conducted. Since in no case was there an indication of a significant loss, it is unnecessary to report all of the analyses. Representative data are given in tables I to III.

The results reported in table I were obtained with Iris leaves that were cut longitudinally and horizontally prior to sterilization. Although these leaves showed considerable bleaching and injury in the vicinity of the cut surfaces, it will be observed that they took up a considerable amount of nitrogen from the medium. In four out of the six leaf samples the recovery of nitrogen agreed very closely with that added; in the other two, where

TABLE I

THE NITROGEN CONTENT OF *Iris* LEAVES AND MEDIUM BEFORE AND AFTER INCUBATION FOR 60 HOURS IN THE PRESENCE OF AMMONIUM NITRATE—EXPERIMENT NO. 1

Leaf sample number	Control leaf halves			Incubated leaf halves				Nitrogen loss or gain
	Nitrogen in medium	Nitrogen in leaf	Total nitrogen	Nitrogen in medium	Nitrogen in leaf	Correction for weight	Total nitrogen	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	60.3	17.1	77.4	50.8	23.8	+2.7	77.1	−0.3
2	60.3	14.6	74.9	52.1	19.6	+1.8	73.5	−1.4
3	60.3	10.5	70.8	50.0	24.2	+0.5	74.7	+3.9
4	60.3	13.7	74.0	52.8	20.9	−0.1	73.6	−0.4
5	60.3	11.4	71.7	49.3	22.5	+0.6	72.4	+0.7
6	60.3	13.8	74.1	59.5	22.7	−0.7	81.5	+7.4

the agreement was not so good, the difference was a gain and not a loss. Obviously the Iris leaves liberated no gaseous nitrogen from ammonium nitrate.

Other similar data with Belamcanda and Narcissus leaves, sterilized prior to cutting, are given in table II. In most cases the nitrogen content of these leaves increased 50% or more during the 60-hour incubation period.

TABLE II

THE NITROGEN CONTENT OF BELAMCANDA AND NARCISSUS LEAVES AND MEDIUM BEFORE AND AFTER INCUBATION FOR 60 HOURS IN THE PRESENCE OF AMMONIUM NITRATE

Leaf sample number	Control leaf halves			Incubated leaf halves				Nitrogen loss or gain
	Nitrogen in medium	Nitrogen in leaf	Total nitrogen	Nitrogen in medium*	Nitrogen in leaf	Correction for weight	Total nitrogen	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
				Experiment no. 2. Belamcanda leaves†				
1	152.0	8.3	160.3	148.0	19.0	−1.2	165.8	+5.5
2	152.0	8.0	160.0	145.0	20.2	−3.1	162.1	+2.1
3	152.0	6.2	158.2	144.0	14.8	−0.3	158.5	+0.3
				Experiment no. 3. Narcissus leaves†				
1	132.3	8.0	140.3	127.2	13.6	−3.9	136.9	−3.4
2	132.3	10.8	143.1	128.2	18.9	−4.9	142.4	−0.7
3	132.3	9.4	141.7	125.0	14.5	−6.8	132.7	−9.0
4	132.3	7.6	139.9	129.5	10.6	−5.9	134.2	−5.7
5	132.3	9.4	141.7	127.8	13.1	−2.0	138.2	−3.5
				Experiment no. 4. Narcissus leaves				
1	67.0	18.9	85.9	58.5	29.1	−0.2	87.4	+1.5
2	67.0	21.7	88.7	55.5	34.2	−1.2	88.5	−0.2
3	67.0	21.2	88.2	56.5	35.2	−4.6	87.1	−1.1
4	17.5	11.3	28.8	15.5	12.8	−0.7	27.6	−1.2
5	17.5	13.4	30.9	16.5	18.9	−2.1	33.3	+2.4
6	17.5	15.8	33.3	17.5	18.4	−1.1	34.8	+1.5

* The pH of the medium after incubation varied from 4.7 to 5.1
† 200 ml. of medium was used for each leaf sample.

There was no loss of gaseous nitrogen from the Belamcanda leaf cultures. In Experiment no. 3 the Narcissus leaves showed a slight loss in all cases, but a repetition of this experiment (no. 4) showed no such losses. The loss in Experiment no. 3 was undoubtedly only apparent, and probably resulted in part from the use of so much ammonium nitrate in the culture medium. A slight error in the nitrogen analysis of a small aliquot shows up as a large value in the final calculations. Where the ratio of leaf nitrogen to medium nitrogen was made very narrow, as in Experiment no. 4, no losses were found.

In table III are shown the nitrogen loss and gain figures where Narcissus leaves were incubated in the presence of ammonium chloride, potassium

TABLE III

THE NITROGEN CONTENT OF NARCISSUS LEAVES AND MEDIUM BEFORE AND AFTER INCUBATION IN THE PRESENCE OF AMMONIUM CHLORIDE, POTASSIUM NITRATE AND AMMONIUM NITRATE

Leaf sample number	Nitrogen source*	Control leaf halves			Incubated leaf halves				Nitrogen loss or gain
		Nitrogen in medium	Nitrogen in leaf	Total nitrogen	Nitrogen in medium§	Nitrogen in leaf	Correction for weight	Total nitrogen	
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
				Experiment no. 5					
1	I	70.0	20.8	90.8	70.0	19.8	−7.0	82.8	−8.0
2†	I	70.0	19.2	89.2	69.5	18.9	+0.6	89.0	−0.2
3	I	70.0	14.9	84.9	67.5	17.1	−5.8	78.8	−6.1
4†	II	55.0	20.8	75.8	49.5	18.5	+6.6	74.6	−1.2
5	II	55.0	23.5	78.5	52.5	25.4	+3.8	81.7	+3.2
6	II	55.0	20.2	75.2	50.0	24.0	+1.5	75.5	+0.3
7	III	62.0	12.5	74.5	56.5	17.1	+1.9	75.5	+1.0
8	III	62.0	17.7	79.7	59.5	20.7	+4.4	84.6	+4.9
9	III	62.0	15.8	77.8	56.0	20.4	+4.9	81.3	+3.5
10	III	32.0	23.4	55.4	29.5	24.4	+5.8	59.7	+4.3
11	III	32.0	21.9	53.9	28.0	26.6	−2.0	52.6	−1.3
12	III	32.0	18.8	50.8	28.5	23.9	+4.6	57.0	−6.2
				Experiment no. 6‡					
1†	I	69.6	20.1	89.7	68.8	21.2	+0.3	90.3	+0.6
2†	I	69.6	24.3	93.9	67.5	26.9	+1.9	96.3	+2.4
3†	I	69.6	18.9	88.5	72.2	18.0	−1.0	89.2	+0.7
4†	III	68.1	21.8	89.9	58.6	26.6	+4.9	90.1	+0.2
5	III	68.1	25.4	93.5	53.9	45.5	−2.1	97.3	+3.8

* Nitrogen source: I = NH_4Cl; II = KNO_3; and III = NH_4NO_3.
† Not sterile.
‡ Incubated for 84 hours instead of the usual 60 hours.
§ The pH of the medium containing NH_4Cl was about 3.5 and that containing NH_4NO_3 was 4.2 after incubation.

nitrate and ammonium nitrate. In several cases the cultures contained bacteria, due in most or all cases to incomplete sterilization by the calcium hypochlorite. These cultures were analyzed, however, and the figures are given here to illustrate the fact that microörganisms did not appreciably affect the nitrogen balance. The few losses shown in table III are offset

by approximately equal gains; hence, it can be concluded that there were no significant losses of nitrogen gas from Narcissus leaf cultures where the three inorganic sources of nitrogen were supplied.

Discussion

In previous discussions of losses of elemental nitrogen as a result of the growth of living organisms, two mechanisms have been particularly emphasized. The first is the reduction of nitrates and nitrites to nitrogen gas by certain aerobic organisms that use combined oxygen instead of molecular oxygen when they are deprived of the latter. Examples of such organisms are *Bacterium denitrificans* and *Pseudomonas fluorescens*.

The other mechanism of loss of nitrogen is by the Van Slyke reaction in which nitrite or nitrous acid reacts with compounds, such as amines or ammonia, to liberate an equal amount of nitrogen from each of the reacting compounds. The reaction is as follows:

$$RCNH_2 + HNO_2 \rightarrow RCOH + H_2O + N_2$$

This reaction is reported (**2, 3, 4, 6, 7, 8, 9, 14**) to occur in cultures of many microörganisms and in higher plants. It presumably proceeds spontaneously in plant cells or culture media where the reacting materials occur in appreciable concentrations. Irving and Hankinson (**6**), for instance, observed a liberation of gaseous nitrogen where Elodea leaves were placed in a medium containing asparagin and nitrate. Nitrite appeared in the cultures and apparently reacted with the amino groups of asparagin. Under normal conditions, where the reacting substances are not present in excess, these authors consider that the reaction does not occur to any appreciable extent.

Burström (**4**) states that the rate of the reaction of nitrous acid with amino acids depends upon the concentration of undissociated nitrous acid. The percentage of the nitrite present as undissociated acid varies from 69 per cent. at pH 3, to 2 per cent. at pH 5.

It is well known that nitrite commonly occurs in plant cells. It may be formed as an intermediary product in the assimilation of nitrate nitrogen to form proteins. It may possibly be formed in plant tissues as an intermediate in the oxidation of reduced forms of nitrogen to nitrates (**11**). It is also taken up through the roots from the soil where it is formed primarily by soil bacteria of the Nitrosomonas group. Nitrite is, however, seldom found in appreciable concentrations in plants. The general idea has been that failure to accumulate in plants is due to rapid transition into amino acids and proteins. This normal assimilation process would, presumably, involve no loss of gaseous nitrogen. If, however, the Van Slyke reaction can occur spontaneously in acid solutions wherever nitrites (or nitrous acid) are present together with certain reduced forms of nitrogen that are also common constituents of plant cells, it may seem difficult to understand why a very considerable portion of the nitrogen used in normal plant growth is not lost to the atmosphere. Mothes explains this reten-

tion by assuming that the nitrite stage is passed so quickly that the nitrous acid has insufficient time to react with the NH_2 groups. BURSTRÖM (**4**) considers this idea untenable and sets up the hypothesis, based in part upon experimental observations, that both nitrate and nitrite are adsorbed upon the colloidal constituents of cytoplasm. These adsorbed groups stand in equilibrium with free ions or acid molecules. The equilibrium would be strongly towards the bound rather than the free acid, and hence little acid would react with NH_2 groups. When nitrate is reduced in normal assimilation, BURSTRÖM would consider that it is more nearly correct to say that it passes the oxidation level of nitrite rather than that it passes through the stage of free nitrite or nitrous acid. Whether this idea is correct remains to be determined, but it is at least in harmony with the results reported here. In addition, it seems to the authors that at the pH of most plant juices there is only a limited opportunity for a reaction between nitrous acid and amino groups because most of the nitrous acid occurs, not as free acid, but as nitrite ions, which according to Burström are not reactive.

Although the present experiments, as well as those of MOTHES (**8**) and MÁZE (**7**) fail to agree with the findings of PEARSALL and BILLIMORIA, (**9, 10**) they are in harmony with the generally accepted idea, based upon years of experimental work, that gaseous losses of nitrogen from healthy green plants are not large. As VICKERY et al (**12**) state, " It would be surprising indeed if it (nitrogen loss) were common in the case of plants grown under more or less normal conditions." It is desired to emphasize, however, that the present studies do not imply that gaseous losses of nitrogen from soils or other biological systems are necessarily small. Under some conditions losses due to denitrification are known to be large, but little is known concerning the importance of the Van Slyke reaction in nature. Additional studies, planned to obtain such information, are now in progress.

Summary

Experiments designed to determine whether green plants liberate gaseous forms of nitrogen from inorganic nitrogen sources during normal metabolism are reported. Leaves of Iris, Belamcanda, and Narcissus were removed from the plant, sterilized in calcium hypochlorite, and floated upon a nutrient sugar-mineral medium for 60 to 84 hours under a bank of fluorescent lights. The forms of nitrogen used included ammonium nitrate, potassium nitrate, and ammonium chloride. Nitrogen determinations were made by the Kjeldahl method of portions of the leaves and media before and after incubation.

Active uptake of nitrogen by the excised green leaves occurred in all cases, but there was no loss of nitrogen during the incubation period. The results, therefore, fail to confirm the observations of Pearsall and Billimoria, who reported large losses of nitrogen where Narcissus leaves were kept under similar experimental conditions.

A brief discussion is given of the possibility of loss of nitrogen from green plants by the reaction of nitrous acid with amines. There is little

to indicate that this occurs to any appreciable extent in the sap of green plants but the importance of this reaction in soils and other media, where microörganisms are active, remains to be determined.

DIVISION OF SOIL MANAGEMENT AND IRRIGATION
BUREAU OF PLANT INDUSTRY, SOILS, AND AGRICULTURAL ENGINEERING
AGRICULTURAL RESEARCH ADMINISTRATION
U. S. DEPARTMENT OF AGRICULTURE
BELTSVILLE, MARYLAND

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THE EFFECT OF THE INTERRELATIONSHIP OF BORON AND MANGANESE ON THE GROWTH AND CALCIUM UPTAKE OF BLUE LUPINE (*LUPINUS ANGUSTIFOLIUS* L.) IN SOLUTION CULTURE

JAMES H. M. HENDERSON[1] AND MARY P. VEAL

(WITH SEVEN FIGURES)

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Introduction

A few years ago widespread interest was shown in the effect of fertilizers containing boron and manganese upon plant growth. These trace elements were used because (1) they exert a marked effect upon plant growth; and (2) they influence very materially the crop yield. The earlier work on the role of boron and manganese has been discussed by BRENCHLEY (**2**), later reviews are those of MCHARGUE and CALFEE (**7**), MCMURTREY (**8**), MINARIK and SHIVE (**10**), SCHUSTER and STEPHENSON (**11**), MEYER (**9**), and THATCHER (**13**). Blue lupine was of interest because it is a relatively new green manure crop in the southern sections of the United States (**6**), although as a plant, blue lupine has been known for hundreds of years. BEAUMONT (**1**) states that the ancient Romans and Germans used lupines in the role of leguminous green manure crops for the purpose of improving soils. It is with this latter purpose in mind that this study was undertaken.

A definite boron deficiency in many soils has been recognized throughout the country in a number of species of crops. As a result, several physiological diseases of plants have in recent years been attributed to this inadequacy of boron compounds in the soils. On the other hand boron may be present in the soils in sufficient amounts, but is rendered unavailable to crop plants, due to some chemical combination, soil reaction, etc.

Manganese is in many instances similar to boron in its effect on plants. THATCHER (**13**) states that manganese has been found by several investigators to be a specific remedy for chlorosis of plants due to excessive lime in the soil. However, its action is not uniform, in that on the same soil, or in the same culture medium, its beneficial effects will be shown by some crops and not by others.

Some investigators have related calcium uptake to the presence of certain minor elements in the soil. MINARIK and SHIVE (**10**) noted that the concentration of boron in the nutrient solution was an important factor for the production of tissues and for the accumulation of calcium in the leaves of the soybean plant. BRENCHLEY and WARINGTON (**2**) have shown that insufficient levels of calcium in solution cultures resulted in definite anatomical abnormalities in the broad bean plant.

[1] Present address: Kerckhoff Biological Laboratories, California Institute of Technology, Pasadena 4, California.

Materials and methods

The culture solution used was that of HOAGLAND and ARNON (**4**), and contained the following principal salts: 0.001 M KH_2PO_4, 0.005 M KNO_3, 0.005 M $(Ca(NO_3)_2 \cdot 4H_2O$ and 0.002 M $MgSO_4 \cdot 7H_2O$. One ml. of a 0.5% solution of ferric tartrate was added to a liter of solution, making a final concentration of 5 p.p.m. One ml. of a minor element solution containing 0.08 p.p.m. $CuSO_4$, 0.22 p.p.m. $ZnSO_4 \cdot 7H_2O$ was added to a liter of final solution. Boron and manganese were added where designated. Boron was added to H_3BO_3 and manganese as $MnCl_2$. Both boron and manganese are expressed in parts per million as the single ions, respectively, i.e., boron as *B* and not BO_3. The experiments reported in this paper contained boron and manganese in concentrations varying from 0.5 p.p.m. to 50 p.p.m.

Seeds which had been sterilized externally in calcium hypochlorite solution (10%) were germinated in sterilized petri dishes containing moistened filter paper. When the seedlings had reached a stage of 2–3 centimeters in length, which took place in from 3–5 days, they were transferred to quart-size Mason fruit jars and placed in holes in paraffined tops held on by the screw-top rings. In all cases the seedlings were grown over a period of fifteen days. There was no renewal of solutions during that period.

In order to determine the effectiveness of boron and manganese on calcium uptake, and since the present micro-methods for determining manganese and boron are less accessible, a method for determining calcium was used. To make analysis for the absorption of calcium, duplicate samples of 500 ml. each were measured from the jars, evaporated to 200 ml. and determined according to the method described by KOLTHOFF and SANDELL (**5**) in the section on "Determination of calcium after precipitation as calcium oxalate."

Experimental results

Data on the dry weight yields and length of stems and roots for experiments are summarized in tables I–III. The data reported for dry weights, length, and milligrams of calcium are averages of the results for each particular group.

The results for the first set of experiments, containing 0.5 p.p.m. of B and/or Mn, show a distinct difference between plants grown in boron and those grown in manganese, especially in the case of the roots (table I). The results for manganese are invariably the same as, or lower than, those for solutions containing neither manganese nor boron. From a similar comparison between plants growing in solutions containing neither boron nor manganese, it is obvious that boron is significantly effective in its influence on growth. Thus, it is logically assumed that it is the presence of boron and not the absence of manganese which causes the difference between groups I–II and III–IV. Since the results in group IV are not ap-

TABLE I

GROWTH OF BLUE LUPINE SEEDLINGS IN SOLUTION CULTURE CONTAINING 0.5 P.P.M. OF B AND MN, AND CA UP-TAKE

Plant group	Solution	Average length in cm.			Average weight in mg.			Average mg. of Ca absorbed	
		Stems	Roots	Total	Stems	Roots	Total	Per group	Per gram dry wt.
I	Control* (41)	14.2	12.0	26.2	96.0	15.6	111.6	7.8	70
II	+ H_3BO_3 (41)	14.0	11.0	25.0	101.8	14.3	116.1	7.5	65
III	+ $MnCl_2$ (41)	13.3	4.7	18.0	91.5	10.4	101.9	7.1	70
IV	− Both (39)	13.1	5.0	18.1	89.5	10.7	100.2	6.6	66

* Control contained both boron and manganese.
Parenthesis ( ) indicates total number of plants per group accounted for in average.

preciably better than those in group III, one can dispense with the idea of manganese toxicity as being the direct cause for the lower result shown in group III in comparison with groups I and II.

The greatest amount of calcium was absorbed when both boron and manganese were present and the lowest amount of absorption took place in plants grown in solution containing neither manganese nor boron (fig. 1 and table I). The absorption of calcium may be due indirectly to the growth of the plant.

In order to test for toxicity and the effect on growth of these elements on

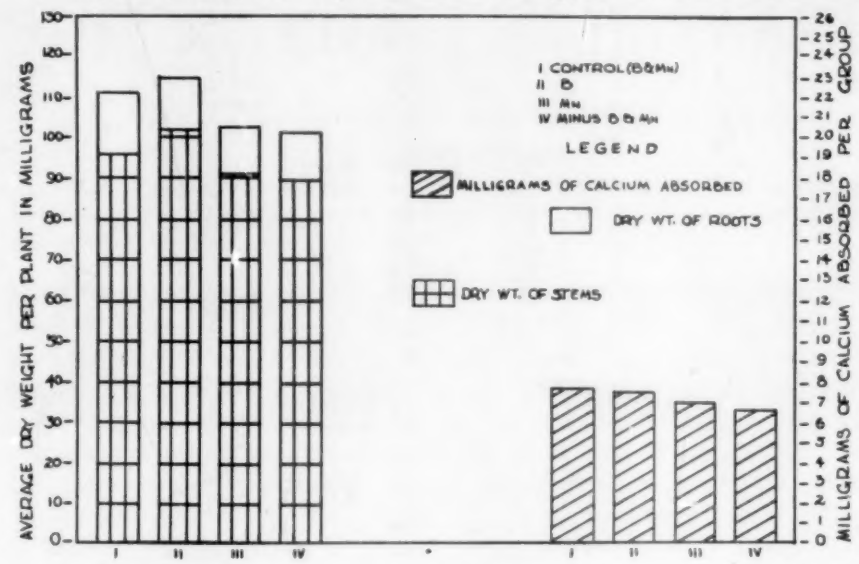
AVERAGE DRY WEIGHT PER PLANT IN MILLIGRAMS
MILLIGRAMS OF CALCIUM ABSORBED PER GROUP
I CONTROL (B & Mn)
II B
III Mn
IV MINUS B & Mn
LEGEND
MILLIGRAMS OF CALCIUM ABSORBED
DRY WT. OF ROOTS
DRY WT. OF STEMS

FIG. 1. Comparison of the growth (dry weight) of blue lupine seedlings with the uptake of calcium in solution cultures, containing 0.5 p.p.m. of boron and manganese.

TABLE II

GROWTH OF BLUE LUPINE SEEDLINGS IN SOLUTION CULTURE AT VARIOUS CONCENTRATIONS OF BORON AND MANGANESE (3.75–7.5 P.P.M.) AND CALCIUM UP-TAKE

Plant group		Concentration		Average length in cm.			Average weight in mg.			Average mg. of Ca absorbed	
		Mn	B	Stems	Roots	Total	Stems	Roots	Total	Per group	Per gram dry wt.
I	(28) (Control*)	3.75	3.75	15.3	14.4	29.7	94.3	16.6	110.9	12.9	116
II	(30)	3.75	None	12.1	11.6	23.7	82.2	13.1	95.3	9.7	102
III	(28)	None	5.0	14.9	13.6	28.5	94.3	16.1	110.4	13.3	120
IV	(30)	5.0	None	11.5	10.3	21.8	81.1	12.6	93.7	8.8	94
V	(28)	None	7.5	14.2	13.9	28.1	92.8	11.7	104.5	14.0	135

* Control contained both boron and manganese.
Parenthesis ( ) indicates total number of plants per group accounted for in average.

blue lupine, the concentrations of these elements in a second series of experiments was increased. In this series of experiments (table II), containing boron and manganese at concentrations 3.75, 5.0, and 7.5 p.p.m., plants receiving 5.0 p.p.m. of boron gave results almost equal to those of plants receiving 3.75 p.p.m. of boron and manganese. When compared with group I, it might be interesting to note that plants receiving 5.0 p.p.m. of boron (group III) showed similar results in dry weight of stems and roots. Plants receiving 7.5 p.p.m. of boron (group V) gave results that were similar to those obtained in groups I and III. There is the possi-

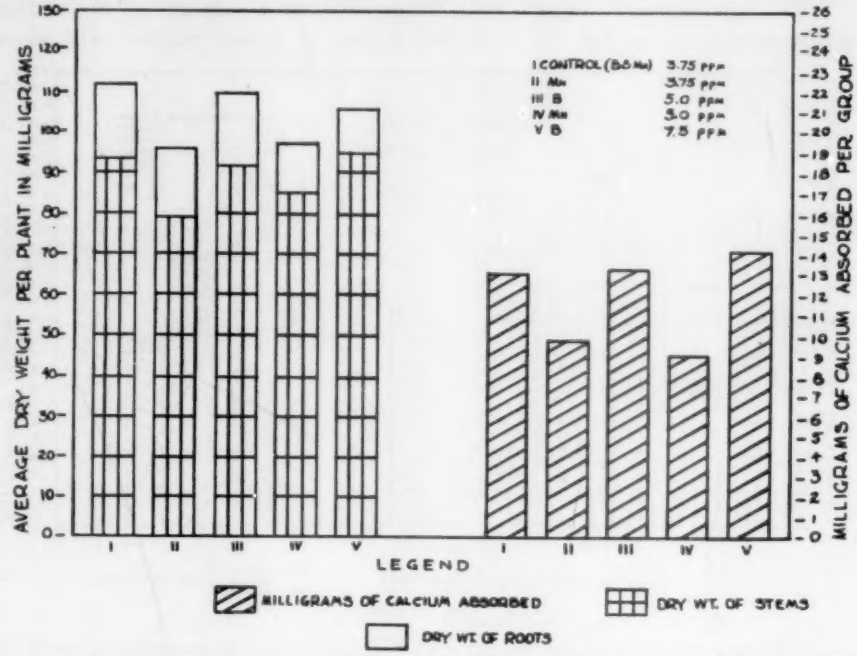
AVERAGE DRY WEIGHT PER PLANT IN MILLIGRAMS
MILLIGRAMS OF CALCIUM ABSORBED PER GROUP
I CONTROL (B.6 Mn) 3.75 ppm
II Mn 3.75 ppm
III B 5.0 ppm
IV Mn 5.0 ppm
V B 7.5 ppm
I II III IV V
LEGEND
MILLIGRAMS OF CALCIUM ABSORBED
DRY WT. OF STEMS
DRY WT. OF ROOTS

FIG. 2. Comparison of the growth (dry weight) of blue lupine seedlings with the uptake of calcium in solution culture, containing boron and manganese at varying concentrations (3.75–7.5 p.p.m.)

bility that here boron may have reached a concentration which is slightly toxic to the roots. Plants receiving 3.75 p.p.m. of manganese (group II) gave low results when compared with plants receiving similar amounts of boron. Plants receiving 5.0 p.p.m. of manganese gave results that were lower than plants receiving 3.75 p.p.m. of manganese. Although there are a number of plants known to have requirements above 1 p.p.m., there are few known which will tolerate concentrations of boron as high as those applied above to blue lupine. MEYER (9), in an investigation on the effect of various concentrations of boron on the development of kok-saghyz, found that the maximum root yields were obtained at 10–15 p.p.m. of boron, although moderately good development of the plants occurred over a consid-

erably wider range of boron concentrations (5–25 p.p.m.). THATCHER (13) states that boron in the form of borax is very toxic to certain crops, if in concentration of more than 3 p.p.m.

The plants in group V containing 7.5 p.p.m. of boron tend to show greater absorption of calcium than those in groups I and III when calculated on a basis of milligrams of calcium per gram of dry weight (table II and fig. 2). Plants receiving 3.75 p.p.m. and 5.0 p.p.m. of manganese show a decrease in the number of milligrams of calcium absorbed as compared with plants receiving similar amounts of boron.

The third series of experiments (table III) for plants grown in solution cultures containing 5.0, 7.5, and 10.0 p.p.m. of boron, respectively, show

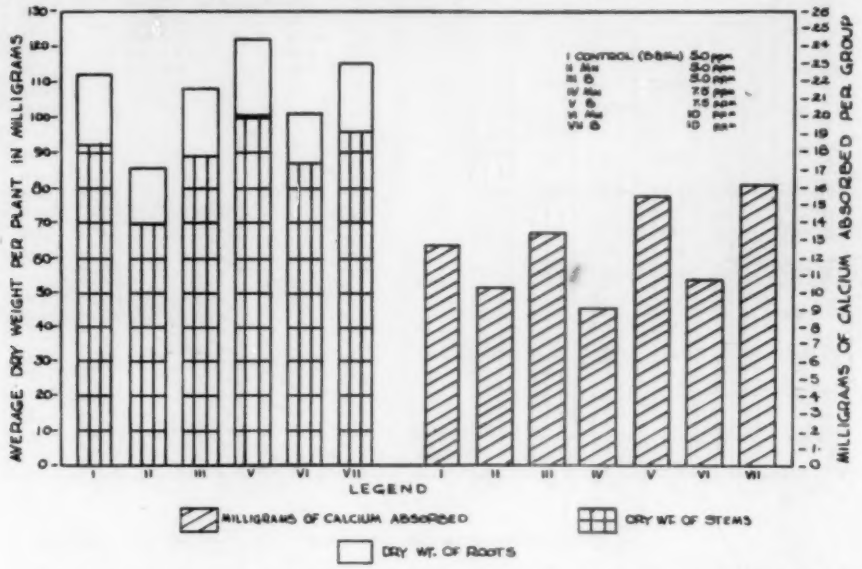
AVERAGE DRY WEIGHT PER PLANT IN MILLIGRAMS
MILLIGRAMS OF CALCIUM ABSORBED PER GROUP
LEGEND
MILLIGRAMS OF CALCIUM ABSORBED
DRY WT. OF STEMS
DRY WT. OF ROOTS

FIG. 3. Comparison of the growth (dry weight) of blue lupine seedlings with the uptake of calcium in solution cultures containing boron and manganese at varying concentrations (5.0–10.0 p.p.m.)

that the lowest results were obtained from plants receiving 5.0, 7.5, and 10.0 p.p.m. of manganese (group II, IV, and VI). It is significant that although the optimum concentration for blue lupine appears to be about 7.5 p.p.m. of B, excellent growth was obtained at higher and lower concentrations. In the first series (table I), in which small traces of boron were added, moderately good results were obtained. Thus it is evident that excellent growth of blue lupine has been obtained in solution cultures over a wide range of boron concentrations.

The data in figure 3 and table III indicate that the highest rate of absorption of calcium was obtained where manganese was lacking and boron was present at a concentration of 10 p.p.m. (group VII). Plants receiving 7.5 p.p.m. (group V) gave results that were almost equal to those of group

TABLE III

GROWTH OF BLUE LUPINE SEEDLINGS IN SOLUTION CULTURE AT HIGHER CONCENTRATIONS OF BORON AND MANGANESE (5–10 P.P.M.), AND CALCIUM UP-TAKE

PLANT GROUP	CONCENTRATION		AVERAGE LENGTH IN CM.			AVERAGE WEIGHT IN MG.			AVERAGE MG. OF Ca ABSORBED	
	Mn	B	STEMS	ROOTS	TOTAL	STEMS	ROOTS	TOTAL	PER GROUP	PER GRAM DRY WT.
I (12) (Control*)	5.0	5.0	20.8	18.8	39.6	92.8	20.1	112.9	12.5	110
II (10)	5.0	None	10.7	8.3	19.0	68.9	16.5	85.4	10.0	117
III (12)	None	5.0	19.9	18.1	38.0	88.8	19.4	108.2	13.4	124
IV (7)	7.5	None	10.3	5.4	15.7	.....	.....	.....	9.1	.....
V (10)	None	7.5	21.5	19.1	40.6	101.3	22.2	123.5	15.8	128
VI (8)	10.0	None	10.2	8.5	18.7	86.6	15.4	102.0	11.0	108
VII (12)	None	10.0	20.4	19.7	40.1	96.0	21.8	117.8	16.3	139

* Control contained both boron and manganese.
Parenthesis ( ) indicates total number of plants per group accounted for in average.

VII. There was a slight decrease in the amount of calcium absorbed by groups I and III compared to V and VII. The lowest amount of absorption of calcium obtained was by plants grown in solution culture receiving manganese. The milligrams of calcium tend to increase proportionately with the increase in the concentration of boron in the solution culture. This seems to indicate that either directly or indirectly boron enhances the uptake and possible utilization of calcium.

A final experiment was set up in order to establish a general toxicity range on blue lupine. The trend of results tends to show that plants grown in solution cultures containing boron would tolerate up to 15 p.p.m. of boron. Plants grown in solutions having a higher concentration of boron tended to show definite toxic symptoms. It might be of some interest to

FIG. 4. Blue lupine seedlings at the end of 15 days taken from solutions containing 5 p.p.m. of boron and manganese (control).

know that the roots of plants grown in solutions containing boron were superior to those grown in solution cultures lacking boron (figs. 4, 5, 6, 7). In most cases there were only a few secondary roots on plants which received small quantities of boron. There were definite gross anatomical abnormalities on plants grown in solution cultures containing no boron. In general when boron was not used there was retardation of growth, a browning at the tip of young leaves and a decrease in their weight.

From the results obtained on the growth of blue lupine seedlings in culture solutions containing manganese, it is evident that manganese when used singly gives results which show no improvement over the results obtained when this element is absent. These results seem to agree with those obtained by SKINNER and SULLIVAN (**12**), who showed that manganese is often ineffective; occasionally injurious. Its effect depends upon the character of the soil.

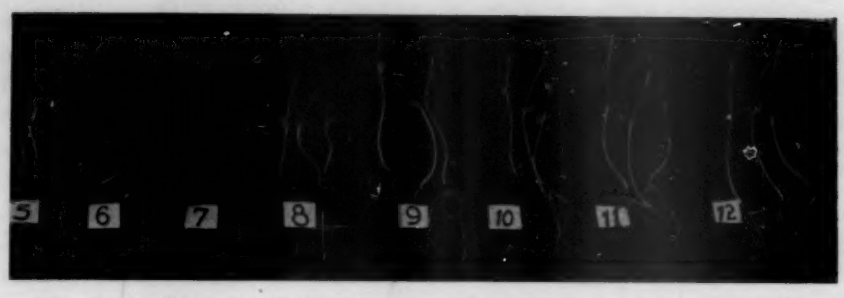

FIG. 5. Seedlings at end of 15 days. 6, 7, and 8 from solutions containing 5.0 p.p.m. of manganese; 9, 10, 11, and 12 from solutions containing 5.0 p.p.m. of boron.

FIG. 6. Seedlings at end of 15 days. 13, 14, 15, and 16 from solutions containing 7.5 p.p.m. of manganese; 17, 18, 19, and 20 from solutions containing 7.5 p.p.m. of boron.

FIG. 7. Seedlings at end of 15 days. 21, 22, 23, and 24 from solutions containing 10 p.p.m. of manganese; 25, 26, 27, and 28 from solutions containing 10 p.p.m. of boron.

Discussion

In most cases plants grown in solution cultures containing boron had more secondary roots than plants grown in solutions lacking this element; there was, also, an increase in both the dry weight and length of stems and roots as compared with solutions containing manganese and lacking boron. Coincident with the increase in growth was an increased uptake of calcium in those plants grown in solutions containing boron. This supports the findings of GLASSTONE (3), who reported that manganese and zinc are not

apparently needed in detectable amounts for the essential growth of tomato roots, although they do play an important part in photosynthesis. It would seem, therefore, that it is the presence of boron and not the absence of manganese which might be responsible for the favorable growth results that were obtained in these experiments.

From another point of view, the use of these three elements (ions), boron, manganese, calcium, suggests a possible antagonism among them. Antagonism may be defined from the point of view of basic cellular reactions as the mutual inhibitory or retarding effect of one ion in solution on the absorption of another, depending on the chemical or physical properties of the ion. Then, when considering the combinations possible in this experiment in relation to elements under investigation, there are three: (1) manganese: boron; (2) manganese: calcium; and (3) boron: calcium. The first, manganese: boron, may be eliminated by the control which showed the best or equal growth to other groups. The second, manganese: calcium, may be eliminated on a comparison of groups containing manganese alone and where both manganese and boron are lacking (calcium is present in all groups throughout). In solutions containing calcium alone the growth was no better than where manganese and calcium were present, thus showing that manganese in no way influenced the effect of calcium on the growth.

Antagonism, in another sense, may be looked upon, especially in the case of toxic ions, as the influence of one ion on another, both of which may be toxic alone, in overcoming this toxicity and stimulating better growth. In this sense then, calcium may appear as somewhat "toxic" (group IV, table I, and fig. 1); but since in solutions containing both manganese and calcium (group III), no better growth resulted, we may again say there is no maganese: calcium relationship here.

However, in the case of boron: calcium we do have a positive response, since where boron is present with calcium or in the presence of manganese, there is better growth. One may cite this as a boron: calcium antagonism, but it would be more accurate to term it boron stimulation. Thus, in either case, increased boron was associated with calcium uptake and possible utilization which resulted in increased growth of the plants. Since in all cases calcium is present in solution, this role must be attributed to the boron in solution. It would seem then, that boron in contrast to manganese aids in the absorption of calcium, the ultimate result being increased growth of blue lupine seedlings. It is not necessarily implied that this result is a direct one. It is quite logical to assume that boron may be acting in one of several ways, primarily as a catalyst.

Summary

1. Blue lupine seedlings grown for 15 days in a Hoagland and Arnon nutrient solution were supplied with boron and manganese at different levels of concentration, ranging from 0.5 to 15 p.p.m.

2. At the low concentration of boron and manganese of 0.5 p.p.m., the

seedlings exhibited marked differences between solutions containing boron and those containing manganese, with the latter of little or no effect on the development of the seedlings. Where neither was present, the results were similar to those of solutions containing manganese alone.

3. At higher concentrations of boron and manganese (3.75–7.5 p.p.m.) similar results were obtained. Calcium uptake corresponded closely with the results, i.e., higher uptake where boron was present.

4. At yet higher concentrations of boron and manganese (5.0–10.0 p.p.m.) growth was similar, indicating a wide range of tolerance for boron. Calcium uptake was equally as good here as for the previous concentrations. Both growth development and calcium uptake were retarded by manganese.

5. For blue lupine optimal concentrations of boron appear to be about 7.5 p.p.m. with a range of tolerance from 0.5 to 15 p.p.m., which is generally higher than most plants will tolerate.

6. Definite anatomical symptoms are shown by plants grown in solution cultures lacking boron. The root system was reduced and top growth retarded generally, especially meristematic tissue. Browning of the tips of the leaves also resulted.

7. An interpretation from the point of view of antagonism between the three main ions concerned (B, Mn, and Ca) is attempted. The conclusion is reached that antagonism as such plays little role, but rather, that boron acts in a stimulatory manner in causing the better growth and development of blue lupine seedlings.

Department of Plant Science
Tuskegee Institute
Tuskegee Institute, Alabama

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CONTRIBUTION OF STUDIES WITH THE ELECTRON MICROSCOPE TO STUDIES OF THE RELATIONSHIP OF CHROMOPLASTS TO CAROTENE BODIES AND CAROTENE BODIES TO VITAMIN A

EDITH A. ROBERTS AND MILDRED D. SOUTHWICK

(WITH THREE FIGURES)

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In 1883, SCHIMPER (**23**) in an article on the development of the chlorophyll grain and plant coloring substances gave an excellent description of sizes and shapes of carotene bodies, in which he stated that they are "sometimes crystals, sometimes granular or amorphous." GUILLIERMOND (**7**) described the chromoplasts as coming directly from mitochondria and stated that they were composed of granules crystallizing out or from transformed chloroplasts. PALMER (**16**) states, "the first yellow plant pigment to be isolated in crystalline form was carotin," and that he "never observed any but granular deposits of carotin in sections of the fresh carrot tissue." HOWARD (**9**) noted a change in plastid shape in fruits during ripening. She found, "In some of the red-ripe fruits the pigment was crystalline in form." She also found, "carrot carotin occurred in both crystalline and granular forms, but free in the cytoplasm." ZECHMEISTER (**37**) maintains that carotene is present in crystals. BEAUVERIE (**3**) finds that the chromoplasts in *Ranunculus acris*, L. petals appear homogeneous, but on application of pressure appear granular. WYCKOFF (**36**) gives a suggestion relative to normal and virus proteins that might be applicable to colored plastids, namely that they might be, "merely modes of disintegration of protoplasm itself." SOBIN and STAHLY (**27**) summarize studies on bacterial carotenoid pigments using methods "adapted to those applicable to the higher plants. Twelve strains of *Staphylococcus aureus* were subjected to pigment analysis. Pigments whose absorption maxima were identical with those of delta-carotene and rubixanthin were found in all the strains studied."

WEIER (**31**) states of carotene that "the pigment appears as small crystalline bodies or it is diffused throughout the cytoplasm. When they are treated with 95% alcohol or absolute alcohol, the carotene crystals may dissolve completely, or a colorless residue which is soluble in chloroform may be left behind." In differentiating cells, carotene was found to be either in small crystalline bodies or diffused throughout the cytoplasm. SCHOPFER (**24**) recognizes that the plastids, because of their enormous surface area are adapted to play an important role and states: "Although the presence of preformed vitamin A in plants is not precluded, no actual study of this problem has been made." STRAUS (**29, 30**) found that carrot carotene occurs as granules or as minute rods. These show the same char-

acters as the protein crystals of vegetable tissues. He finds that carotene consists of 60% proteins, 35% lipids and greatly resembles bacteria in appearance. He finds also that it possesses motility, multiplies by fission and agglutinats at a pH of about 4.5. By a series of centrifugations he resolves the "Kleinste Körnchen Durchmesser etwa 0.5 microns." MAYNARD and BEESON (**12**) find that variation in "Provitamin A: Carotene" may be due to direct exposure to light.

More and more kinds of carotene are described each year. PORTER and ZSCHEILE (**18**) give a list of these from Lycopersicon species and strains. These "have been separated by chromatography (MgO Super-Cel, 50–50) and characteristic absorption curves have been presented for each." ZSCHEILE (**38**), ZSCHEILE, HARPER, and NASH (**39**) and ZSCHEILE and PORTER (**40**) use chromatographic and spectrophotometric analysis in the determination of vitamin A. STRAIN (**28**) finds that "separation of vitamin A from the carotinoids is important in biological investigations and may be accomplished by chromatographic adsorption." PETERING, BENNE, and MORGAL (**17**) and NASH and ZSCHEILE (**15**) find that the absorption curve of zeta-carotene is similar to that of lycopene.

HEILBRON, JONES, and BACHARACH (**8**) state that "vitamin A itself has never been found in vegetable products," though "the chief ordinary sources of vitamin activity are the green leaves of plants and root crops such as carrot which contains carotene." They also give "The Vitamin A and Carotene Contents of Some Important Sources" as follows:

SOURCES	VITAMIN A	CAROTENE	TOTAL VITAMIN A ACTIVITY
	mg. per 100 g.	*mg. per 100 g.*	*I.U. per g.*
CARROT	none	2 to 10	(35 to 200)
LIVER OIL (HALIBUT)	(800 to 15,000)	none	20,000 to 360,000
TOMATO	none	14 to 36	(300 to 700)

As for vitamin A, the CARR-PRICE (**4**) method of identification has met with certain objections. SOBEL and WERBIN (**26**) question its value, as well as do JOHNSON and BAUMANN (**10**) who find a wide variation in the blue. BAUERNFEIND and BAUMGARTEN (**1**) found that the crystalline carotene preparations of different purity showed the T pigment and "that under the conditions encountered, beta-carotene may undergo isomerization or some other spontaneous change." MENKE (**13**) found carotinoid-protein compounds in plastids similar to chlorophyll-protein compounds. FRENCH (**5, 6**) found a carotinoid-chlorophyll protein complex to exist in certain bacteria, while MITCHELL (**14**) found that vitamin A or A_2 were in the form of conjugated-carotenoid proteins and constituted "an integral part of the retinal mechanism concerned with light detection and discrimination." WILLIAMS (**32**) finds "distribution of vitamin A perhaps the most erratic of any of the known vitamins," while SHANTZ, EMBREE, HODGE, and WILLS (**25**) note "the apparent ability of vitamin A_2 to re-

place vitamin A_1 in general body functions." BAXTER, HARRIS, HICKMAN, and ROBESON (**2**) report that the "vitamin A gave an ultraviolet spectrum apparently identical with that of the crystals, but it proved refractive to crystallization" and ROBESON and BAXTER (**22**) report that from the so-called "non-crystallizable vitamin A" an unrecognized geometric isomer of vitamin A has been isolated and call it "neovitamin A." WILSON *et al.* (**33**) claim the fact "that the chemical and bioassay methods of (carotene) do not always agree and must be due to the inability of the test animal to absorb all of the carotene in the supplement," and WITH (**34, 35**) asks, "Do carotene and A-active carotenoids act as provitamin A or do they unfold their biological action in the organism without previous transformation into vitamin A?" In the work of KOEHN (**11**) "support is given to the theory that conversion of beta-carotene into vitamin A in vivo is by fission of the molecule, yielding two molecules of vitamin A and not by an oxidative reaction that inactivates one-half of the molecule."

This most recent work brings to a focus certain questions still unanswered as to the relationship of vitamin A to carotene. This may have a bearing on (1) the "discovery" of new forms of vitamin A and carotene, (2) the dissatisfaction with methods of their determination, (3) the origin of vitamin A and (4) the "synthesis" of vitamin A. It is with these questions in mind that we are undertaking to see what contribution studies with the electron microscope make to the relationship of chromoplasts to carotene bodies and carotene bodies to vitamin A.

Materials and methods

It is recognized that the main contribution of observations made with the electron microscope relative to chromoplasts, carotene bodies and vitamin A will be, of necessity, that of information as to the relationship of structure and size of these bodies. Knowledge gained of the enormous surface areas involved as evidenced by the size will contribute to the effect each have upon their respective functioning in the plant or animal body.

Chromoplasts and carotene bodies are known to be present in plant members throughout the plant kingdom, but it is necessary, from the standpoint of space, to limit the illustrations in this paper. Carotene bodies from *Daucus carota*, L., var. *sativa*, DC.; *Cuscuta arvensis*, Beyrich.; *Mycobacterium Phlei*, Lehmann and Neuman; male gametes of *Allomyces* sp.; *Rosa rugosa*, Thunb.; *Sorbus americana*, Marsh.; *Solanum pseudocapsicum*, Linn.; commercial "crystalline" vitamin A, "beta-carotene" and "alpha-carotene" are shown. However, sufficient electron micrographs already taken would evidence that the interpretations made of these given here are applicable to the chromoplasts, carotene bodies and vitamin A of all plant forms from bacteria to the higher plants.

In all instances aseptic precautions were taken in handling the material, which was mounted on a cellulose acetate film and the pictures were taken with the RCA Electron Microscope, Model EMC. Though the use

of electron micrographs in scientific literature is rapidly increasing, it seemed as if supporting Kodachrome photomicrographs, where possible, might aid in the presentation of the material. When the electron micrograph observations are supported by photomicrographs in Kodachrome, interpretations may be evaluated with a greater feeling of confidence. While various interpretations may be given, as with all new methods, it is only by their presentation that they can receive evaluation.

Results

The relationship of chromoplasts to carotene bodies to vitamin A, as interpreted here, was first presented at the A.A.A.S. Meeting in St. Louis in 1945 by ROBERTS (**20**). At this time, protoplasmic structural and functional entities of plant cells and their contents were shown. These were smaller in size than any reported to that date, namely less than 100 ängstroms in diameter. These units, whether in an anabolic or catabolic process appeared to be protoplasmic and, hence, functional in the plant. Additional information was given in reports by ROBERTS (**19, 21**).

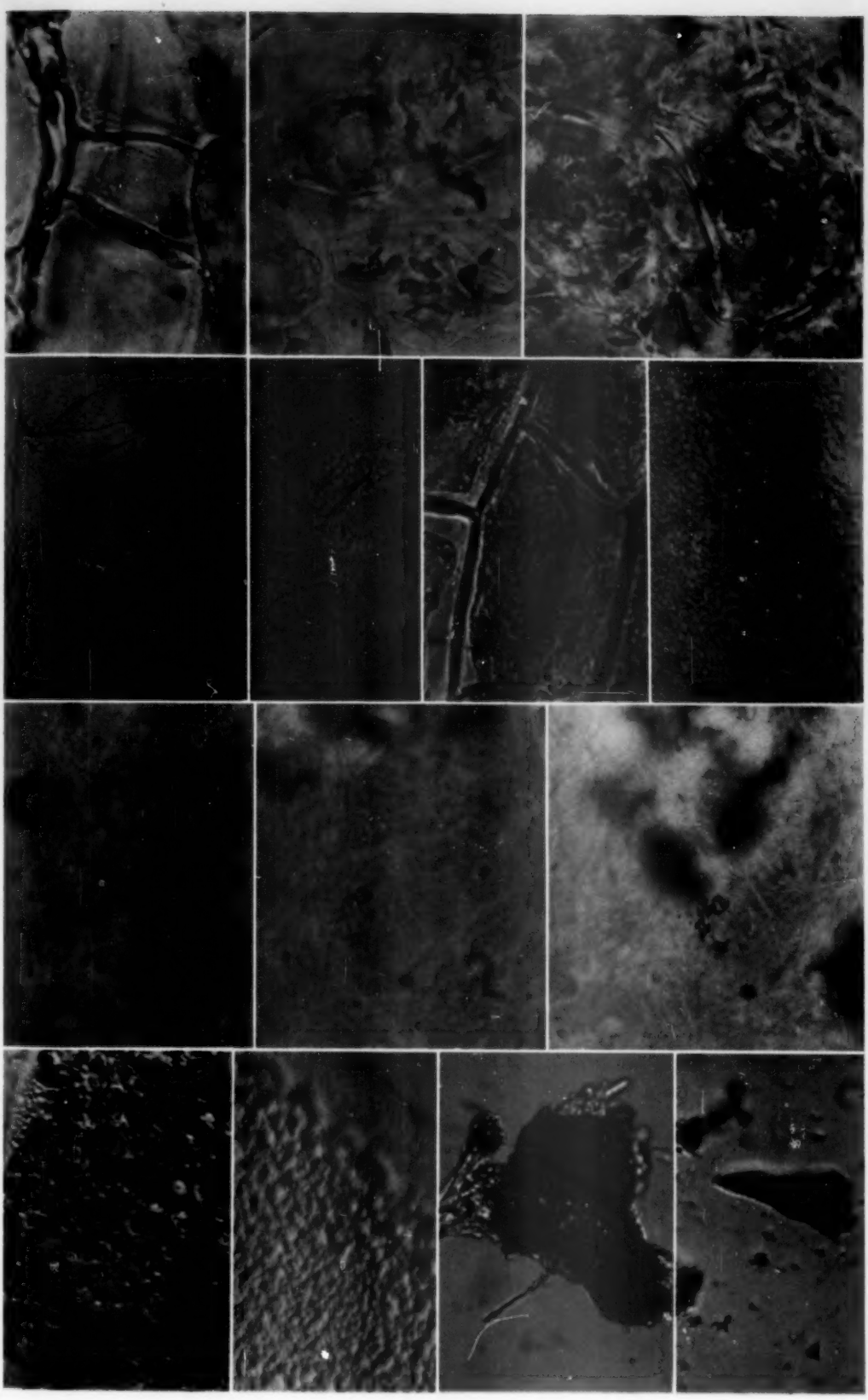
The chromoplast of the carrot has very frequently been used as a source of the many carotenes. It is the one from which it has been said that beta-carotene has been converted into vitamin A. Figure 1A is of a root cell of *Daucus carota*. The very small roundish bodies are seen in the protoplasm and a ribbon-like mass appearing as part of the wall itself. These ribbons are commonly seen in various forms and lengths in the carrot root cells and can be seen being torn off from the wall itself. They appear to be fragments, previously laid down in the cell wall, which in the older cells, break off and float in the cell sap. These and the interchromoplastid strands, which are themselves colored or pigmented, account for the multitude of shapes and sizes of what has often been illustrated as "carotene crystals." These "crystals," when treated with Ruthenium red give evidence of pectinaceous substances being present even in the smallest particles. The carotene bodies of *Rosa rugosa* are shown in figure 1B. The chromoplasts, which are composed of irregular masses and particles of less than a micron in size, are shown in each plastid. These, when treated with Ruthenium red, give the color attributed to pectinaceous substances and when treated with I_2KI and H_2SO_4 give the color attributed to "cellu-

FIG. 1. Top row, left to right: *A*. Small, roundish bodies in ribbon-like mass in root cell wall of carrot. *B*. Carotene bodies in *Rosa rugosa*. *C*. Chromoplastids in *Solanum Pseudo-capsicum*.

Second row, left to right: *D* and *E*. Roundish bodies and needle-crystal-like carotene bodies 2 to 3 entities wide in the center in *Sorbus americana*. *F*. Particulate carotene bodies crushed and separated into small particles.

Third row, left to right: *H*. Dried carrot carotene bodies treated with benzene. *I* and *J*. Same resolving into bodies less than one micron in diameter in size.

Fourth row, left to right: *K*. Commercial alpha-carotene "crystals," showing nugget-like masses. *L*. Same crushed. *M*. Commercial "crystalline" beta-carotene. *N*. Commercial "crystalline" vitamin A.



lose." The chromoplastids in *Solanum Pseudo-capsicum* (fig. 1C) show the chromoplastids made of bodies of similar one micron size and very clearly show the small colored bodies in the inter-plastid plasmic strands.

In figure 1 D and E, *Sorbus americana*, the carotene bodies are of two distinct types, the roundish masses and the somewhat needle-crystal-like bodies, each of which are again made of one micron diameter bodies, the crystal-like ones being of two to three entities wide in the center, tapering to the width of one at the tips. When the pigment is extracted, the bodies show even more clearly. The carotene bodies of *Cuscuta arvensis*, (fig. 1 F and G) are most particulate in character, each particle standing out in a firm rounded mass. The larger masses are easily crushed and when this is done, the seemingly firm masses separate into smaller and varying sized masses, the intensity of the yellow color varying from the deep yellow to a pale yellow, depending upon the size of the mass, and becoming very pale yellow as the size reaches the resolution of the microscope equipment, namely $\frac{1}{3} \times$ Periplan, $10 \times$ eyepiece and water immersion objective. While these bodies can be easily separated into small particles by simply crushing the carotene bodies of the carrot, they can also easily be seen to separate into minute entities by adding a drop of benzene to a dried chromoplast. In less than an hour, as seen in figure 1 H, I, and J, a large mass is resolved into bodies less than a micron in diameter in size, the color remaining present in the smallest bodies.

The carotene bodies in *Mycobacterium phlei* and those of the male gametes of Allomyces, sp. and other forms observed fall into the same type as given above, namely they appear as masses obviously particulate in type, or fragmented. The latter is most often referred to as "crystals." In all instances, these particulate bodies contain "cellulose," pectinaceous substances and a yellow pigment.

Figure 1 K and L, are of the commercial carotene "crystals" which, with centrifuging, are separated from large "crystals" (nugget-like masses) into smaller and smaller masses, even to tiny bodies which still show the yellow color. Commercial "crystalline" beta-carotene (fig. 1M) shows the same particulation, while commercial "crystalline" vitamin A (fig. 1 N) is composed of exactly the same bodies as in the dodder or carrot before they were built into larger and denser masses.

Thus, with the Kodachrome photomicrographs alone, one is led to infer that it is because of the more conspicuous and larger bodies, due to the greater masses, that the smaller pigment-containing bodies are unnoticed. They can be seen, however, if attention is called to them and are especially easily demonstrated in the Jerusalem Cherry and in dodder, as well as in the carrot. While the support of the electron micrographs by the Kodachrome photomicrographs has been mentioned, it might in turn be suggested that the electron micrographs support the interpretations of the Kodachrome photomicrographs.

In preparations which were made of the carotene bodies of the carrot

(fig. 2 A) the pigmented network masses, which were one micron in diameter in the photomicrographs, proved in the electron micrographs to be composed of denser masses held together in the same manner as the one micron particles in the Kodachromes. There is the same repetitive pattern showing the netted mass to be composed of bodies of diminishing sizes from the 10,000 Ångströms to smaller and smaller masses, until in the thinner portions they are less than 160 Ångströms in diameter.

In figure 2 B, the masses are more separated and the inter-protoplasmic strands are still apparent in the smaller masses. The carrot bodies are less dense and the network shows more distinctly, but basically the structure and sizes are the same. Figure 2 C shows the smallest masses discernible with the electron microscope and of which all the bodies are composed, but which in the living, moist material has been spread into a thin mass of pale yellow particles less than 100 Ångströms in diameter. Commercial alpha-carotene was treated in a like manner. "Crystalline" alpha-carotene was crushed and mounted on a cellulose acetate electron microscope film. The same particulate character was shown as in the fresh carrot. The dark orange—the color being somewhat darker, due possibly to greater concentration and greater dehydration—of the "crystal" changes to pale yellow in color as the particles are separated farther apart and spread out into a thin film of smaller and smaller units, which are exactly like, in size and color, the particles seen in the carrot. This was true of all the carotene bodies of the various plant organisms investigated. They prove to be similar in size and shape to the commercial "crystalline" carotene and both the natural forms and the commercial "crystals" appear to be protoplasmic entities to which the pigment is adsorbed. The so-called "crystals" only being in a very dehydrated form. The same was true of beta-carotene "crystals."

Figure 3 A is an electron micrograph of "crystalline" vitamin A. The "crystals" were both crushed and water "dissolved." They show that the material, as to sizes and shapes, is composed of material comparable to the carotene bodies. This is clearly shown along the edges of the "crystal" as it is being broken up. Figure 3 B is an electron microscope picture of a crushed "crystal" of vitamin A after rehydration, showing that it is composed of a network of entities less than 166 Ångströms in diameter.

Figure 3 C shows the "crystal" vitamin A crushed a bit finer. The network of particles is denser than in the living material, as in figure 2 C, due to greater dehydration, but does show the strands of material which hold them together. These masses mounted on the film on the electron microscope screen showed a faint yellow color under the light microscope. Also in figure 3 C the "crystals" of vitamin A are seen in various stages of breakdown. The larger dehydrated masses are too dense for electron penetration and hence, appear almost solid, as in figure 3 A, but as the material spreads out, the areas become thinner and the very thin areas

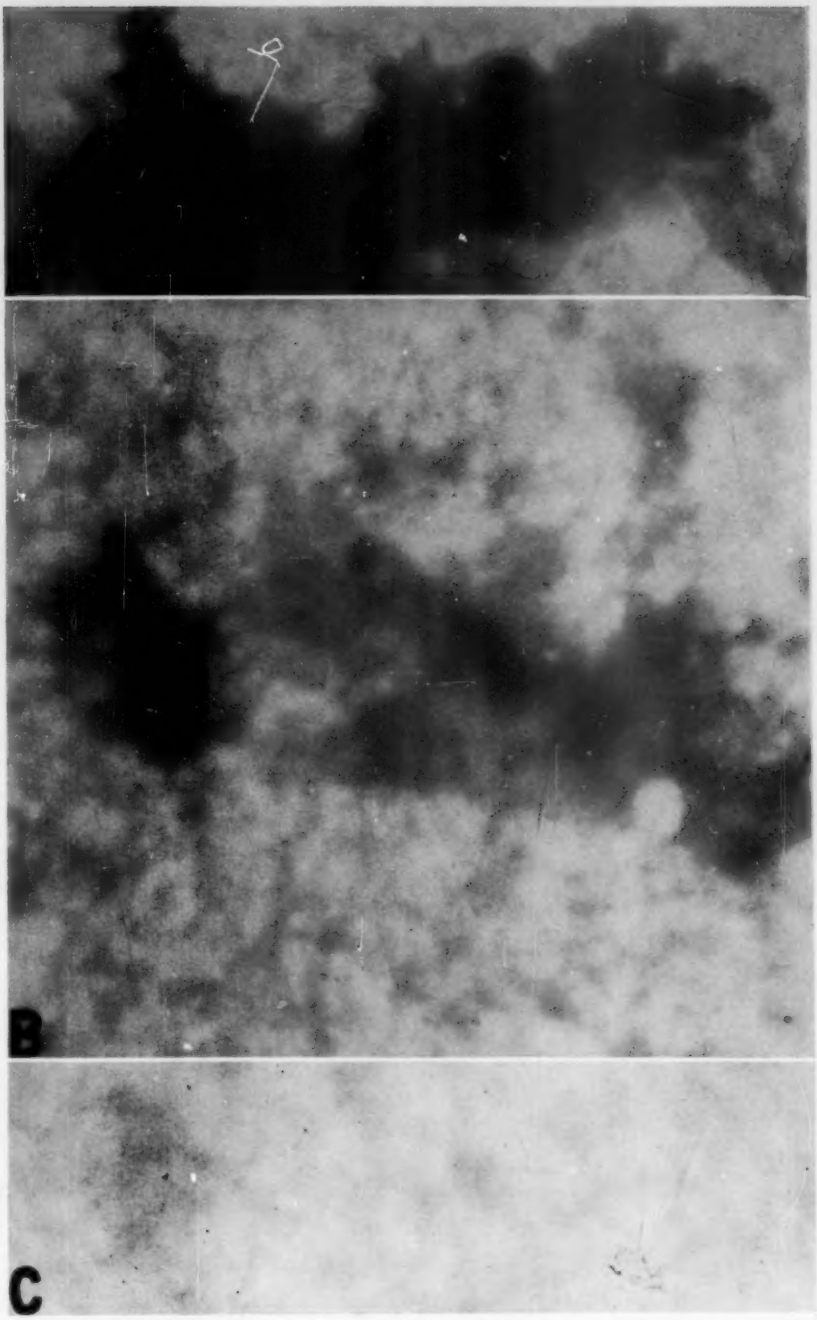


FIG. 2. Electron micrographs × 60,000. |———— 1 micron ————|. *A*. Fresh carrot carotene bodies showing repetitive pattern of network masses. *B*. The same as *A*, but spread more thinly, showing very distinctly the inter-connecting strands. *C*. Shows the smallest connecting masses of the fresh carotene bodies discernible with the electron microscope.

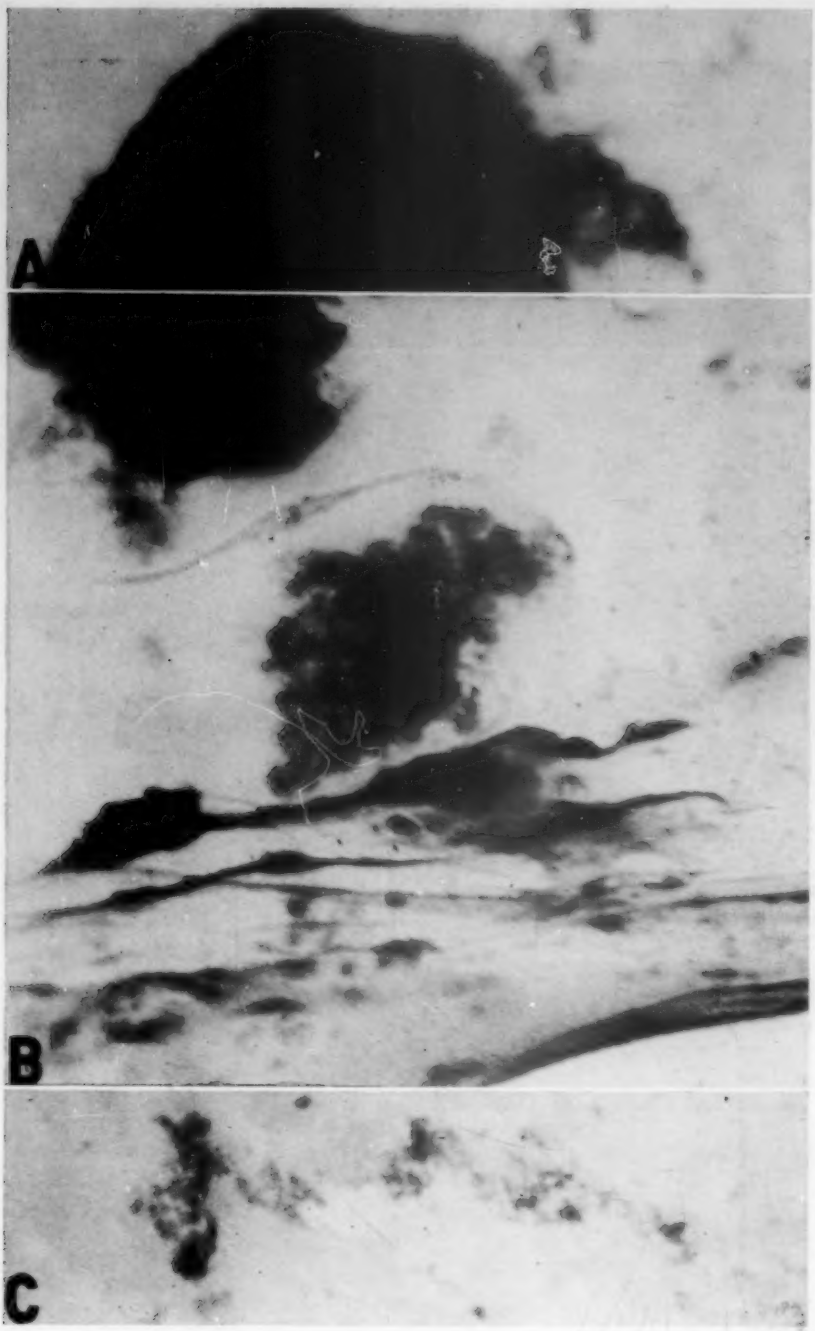


FIG. 3. Electron micrographs × 60,000. |———— 1 micron ————|. *A*. Crushed "crystalline" vitamin A, showing the particulate character comparable to the carrot carotene bodies. *B*. The same as A, but after rehydration, showing the network of entities. *C*. Showing the network of particles denser than in the fresh carrot (fig. 2 *C*), due to greater dehydration, but of comparable size and with comparable connecting strands.

are as penetrable as the bodies of living material in figure 2 C. It will be noted that the reticulate structure is the same and the ultimate size is of the same order as they are in the carrot chromoplasts, namely somewhat less than 160 Ångströms in diameter.

Discussion

The chromoplast of the cell in various members of the plant kingdom is shown to contain pectinaceous substances and so-called "cellulose" material. These masses of protoplasm are seen in the light microscope to be composed of a reticulum, the smallest entities of one micron in diameter being held together by fine strands of protoplasm. The fact that with the aid of the electron microscope these chromoplasts are found to consist of connected entities, repeatedly smaller and smaller, but the units being composed of the same reticulate matter forming a repetitive pattern, indicates that they, too, are of the same protoplasmic nature as the larger protoplasm bodies and this appears to be true even when particles are less than 100 Ångströms in size. They are still very small reticulate masses of protoplasm and by no means crystalline in character. The denser masses of the network (reticulum) give the appearance of granular bodies and in the smaller entities, the inter-protoplasmic strands are more difficult to see and smaller entities might easily be considered separate entities or granules. The matrix can be treated so that the denser masses of the reticulum separate and the body appears, and undoubtedly is, disintegrated because of the treatment. The smallest observable entities—for there are inter-protoplasmic strands too fine to be demonstrated at the magnification used—are present in the very young living cells, so that it would seem that the building up of protoplasm from the smaller and smaller protoplasmic masses is to be seen in these young cells, while the breakdown is taking place in the older cells. This is easily seen in the very young and old cells of carrot root. Insofar as structure can be discerned with the electron microscope, the entities of 100 Ångströms in diameter, at least, are protoplasmic in character and not crystalline. The enormous surface areas produced by these repetitively reticulate bodies must play a very important part in the retention of the pigments upon the surfaces of these less than 100 Ångström masses of protoplasm. Even at the 100 Ångström mass, the beginning of the formation of the protoplasm entities is not reached and it would seem that there are still smaller masses, or protoplasmic bodies which, to date, cannot be demonstrated even with an electron microscope.

It would appear, however, that vitamin A bodies occur in the anabolism of carotene bodies and that these very smallest 100 or less Ångström units are what are known as "vitamin A." Though it is known that vitamin A is made by the plant, the fact that it has never been demonstrated is, doubtless, due to the size of the vitamin A bodies, for if vitamin A appears to be a breakdown product of a carotene, then it seems reasonable that

these bodies are the ones which take part in the anabolism of carotene bodies.

It is claimed that one of the functions of vitamin A is that of stimulating the building of cells. This would mean the building of protoplasm. If the vitamin A bodies are, themselves, protoplasm, they certainly would contain the essential materials for the formation of more protoplasm and, in turn, more cell walls in plants. The resemblance or identity of the entities with so-called "cellulose" bodies raises the question of the relation of the pectinaceous and "cellulose" content of protoplasm itself to the building of the cell walls. The water holding capacity of these 100 Ångström entities with their surface forces and the resistance to dehydration certainly raise many questions relative to the function of vitamin A.

Summary and conclusions

The electron micrographs of materials from various members of the plant kingdom from the bacteria to higher plants and of commercial "alpha-carotene," "beta-carotene" and vitamin A, supported by photomicrographs suggest that vitamin A represents a step in the anabolism as well as the catabolism of carotene bodies and that the carotene bodies, likewise, represent steps in the anabolism and catabolism of chromoplasts.

The chromoplasts are built up of protoplasmic entities less than 100 Ångströms in diameter. While in the less than 100 Ångström size, they function as the several vitamins in the A group and as these are built into larger units, the color becomes more distinct and they are then recognized as the various carotene bodies, such as alpha-carotene, gamma-carotene, and delta-carotene, depending upon the size of the masses—the so-called "carotin crystals" of various types.

The recognized close relationship of carotene to chlorophyll would give support to the belief that the carotene or vitamin A quality is in material adsorbed to the many surfaces of these small protoplasmic entities and that the varying sizes of the protoplasmic entities determines whether they are functioning in the manner of the vitamin A group or in the carotene group or are recognized as the still larger protoplasmic mass in the chromoplasts.

In the plant, the chromoplasts are the more apparent bodies, but by careful observation, even with the light microscope, entities of one micron in dimension can be seen forming part of the chromoplast protoplasmic mass in the cells and, as in the chloroplasts, the small entities have considerable color, the color of the chromoplast being basically the same as that of the color in these one micron masses. In the cell sap, these small bodies can be discerned and, as they divide and grow, the color becomes more evident as the mass increases until the varying sized chromoplasts are discernible. The varying amounts that exist at any one moment in cell contents, whether it be in the anabolic or catabolic stage, and the proportion of sizes present varies enormously. Alpha-carotene "crystals,"

beta-carotene "crystals" and vitamin A "crystals" can all be shown to be composed of structural bodies identical with the entities of which the chromoplasts are composed, the size and structure of the vitamin A being the smallest discernible without the use of the electron microscope, the more dehydrated commercial bodies being more discernible than the material in the living cells. These facts may well account for the many vitamin A determinations being most erratic and for new types of carotene and vitamin A being "discovered," all depending upon the methods of determination and the anabolic or catabolic status of the plant material being used. As in the more recent "synthesis of vitamin A," the synthesis has been carried on in the plant material itself, man merely extracting the particles corresponding to the vitamin A in size and structure already produced by the plant.

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DEPARTMENT OF PLANT SCIENCE
VASSAR COLLEGE, POUGHKEEPSIE, NEW YORK

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BRIEF PAPERS

A FACTOR METHOD FOR THE AREA OF TOMATO LEAVES

CHARLES J. LYON

(WITH ONE FIGURE)

Received July 7, 1948

It is a fundamental principle of geometry that the areas of similar plane figures vary as the square of one dimension. It is obviously difficult to apply this principle in computing the area of such a dissected surface as the pinnately compound leaf of the garden tomato. The difficulty has been overcome for practical purposes by determining the simple mathematical relationship between the overall length of the leaf and the total area of the leaflets. The form of the leaf seems to remain essentially constant, regard-

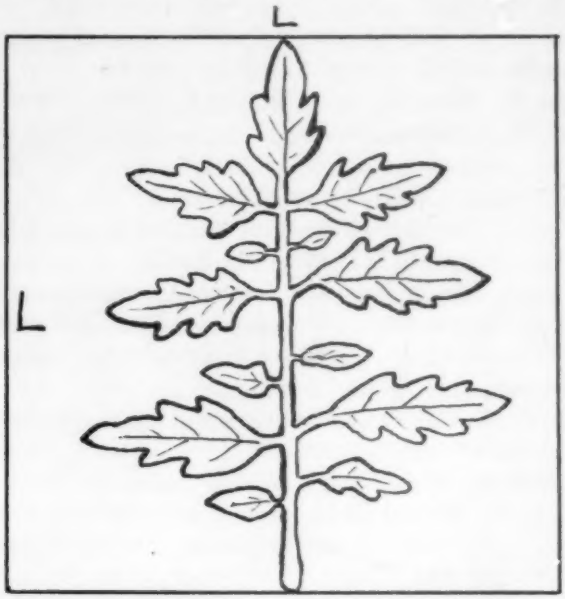

FIG. 1. Leaflet area = 0.1551 (leaf length)^2.

less of its size. The method is sufficiently accurate for use in following the growth of the tomato when it is used as a test plant in fertilizer tests or other growth studies.

The method was developed with the Bonny Best variety of tomato, grown in greenhouse pot culture. The length of leaf was measured from tip to base of petiole, using a flexible foot rule with readings to one-eighth inch. The leaflet area of each leaf was then determined by placing the leaf flat on white paper and outlining each leaflet in pencil. The paper

was cut along the pencil lines and the group of leaflet tracings weighed to the nearest 0.01 gm. The area of leaflets was then obtained by dividing the weight of the paper tracings by the weight of a unit area of the paper, essentially uniform in thickness.

After the areas of 25 such leaves, ranging in length from $3\frac{3}{8}$ to 12 inches had been thus measured, the factor K in the equation $K = \frac{\text{Area}}{\text{Length}^2}$ was computed for each of the 25 leaves. The mean value of this factor proved to be 0.1551 ± 0.0053. Since the standard deviation of this computation is less than $3\frac{1}{2}$ per cent. of the mean, this leaf area factor is therefore fairly reliable.

Although the area of any one leaf would normally be computed by squaring the length and multiplying by 0.1551 (since $A = KL^2$), a nomogram would be constructed and used for extended measurements of leaf areas. Presumably the factor K will vary somewhat for different varieties of tomato, thus requiring a preliminary computation from some actual measurements of length and area. The extension of the method to the simple or compound leaves of other plants would be a simple matter.

DARTMOUTH COLLEGE
HANOVER, NEW HAMPSHIRE

A TECHNIQUE FOR INTRODUCING RADIOACTIVE PHOSPHORUS INTO THE GRAIN OF GROWING CORN

H. G. M. JACOBSON

Received March 6, 1948

Corn grain containing a relatively high concentration of radioactive phosphorus was needed for a poultry feeding investigation by the Poultry Husbandry Department at the University of Connecticut at Storrs. A gravel nutriculture procedure was decided upon for growing the corn. Uniformity of maturity was desired so a hybrid field corn (Connecticut 520) was selected as the corn to be grown for this experiment.

Methods

PHYSICAL EQUIPMENT.—Two water-tight boxes having a surface area of eight square feet and a depth of eight inches were filled with gravel to within two inches of the tops of the boxes. The gravel was obtained locally from the C horizon of a Manchester soil. It contained a high percentage of gravel-sized sandstone fragments mixed with some granitic material. After sieving the gravel through a half-inch mesh screen, it was washed over a screen having six meshes per inch. The material going through the six mesh screen was discarded. Each box was filled to a depth of six inches with the screened gravel. The boxes were tilted toward one corner so that they could be thoroughly drained by means of a syphon. Each box required 42 liters of solution to submerge the six inches of gravel. The nutrient solution for each box was stored in separate twelve gallon covered carboys. This solution (table I) was forced once daily with an air pump from the carboys to the culture boxes and immediately returned to the carboy by a syphon. The solutions were maintained at a definite volume by the addition of tap water at intervals when necessary.

PROCEDURE.—On February 1, 1947 three rows of corn were planted 2 inches apart in the row in each culture box. The rows were spaced about

TABLE I

NUTRIENT SOLUTION. The chemical composition of the nutrient solution made up to 42 liters was as shown. Minor elements were added to the nutrient solution as follows: Ferric chloride, 1.875 grams; Boric acid, 0.125 grams; Copper sulphate, 0.125 grams; Potassium permanganate, 0.190 grams.

COMPOUND	TOTAL WEIGHT	WEIGHT OF ELEMENTS (grams)					
		N	P	K	Ca	Mg	S
KNO_3	48	6.7	0.0	18.6	0.0	0.0	0.0
$Ca(NO_3)_2 \cdot 4H_2O$	46	5.4	0.0	0.0	7.8	0.0	0.0
$Ca(H_2PO)_2 \cdot H_2O$	42	0.0	10.5	0.0	6.7	0.0	0.0
$MgSO_4 \cdot 7H_2O$	25	0.0	0.0	0.0	0.0	2.5	3.2
Total grams	161	12.1	10.5	18.6	14.5	2.5	3.2
	ppm	288	250	443	345	60	76

12 inches apart. The plants were later thinned to 5 plants per row spaced about 7 inches apart per plant. The corn was illuminated before sunrise and after sunset to simulate a July length of day. Two fluorescent lighting fixtures each having two fluorescent day-light bulbs four feet long were used for this purpose. Insects were controlled by naphthalene vapor at weekly intervals. A small blower was used at tasselling time to insure proper pollination. The nutrient solution was tested bi-weekly and the necessary chemicals were added to maintain a balance of the elements.

The corn started to tassel April 15 and the first silk appeared April 25. On May 9 the kernels were in the milk stage and by June 10 when harvested they were well matured. The height of the corn stalks averaged nine feet at maturity. Approximately 10 pounds of good quality grain were harvested from the two culture boxes (about 390 bu. per A).

Radioactive Phosphorus Introduction.—The nutrient solutions were replaced with tap water May 5 and 166.25 milicuries of radioactive phosphorus in the form of KH_2PO_4 were added to the water of each culture solution. The concentration of KH_2PO_4 was equivalent to 38.0 gms. of KH_2PO_4 and gave a concentration of 189 ppm of phosphorus and 260 ppm of potassium to the solution. On May 7 full-rate nitrogen and potassium was added to the nutrient solutions. One-half rate $Ca(H_2PO_4)_2 \cdot H_2O$ was added May 15 and another one-half rate May 27. No more chemicals were added for the duration of the growing period.

Results

The harvested corn showed a strong radioactivity in all parts with the grain being the most active of all. On June 13, 100 mg. of the ground corn averaged 2800 counts per minute. On June 16 calcium phytate isolated from the corn averaged 47,040 counts per minute for a 100 mg. sample, indicating an excellent concentration of radioactivity in the phytin phosphorus fraction, as reported to the author by Dr. E. P. Singsen, Acting Head of the Poultry Husbandry Department of the University of Connecticut, for whom the corn was grown.

Summary.—Employing a gravel nutriculture procedure, corn of high quality and yield was produced. Radioactive phosphorus was introduced into the nutrient solution when the corn was in the early milk stage of kernel production. The mature corn grain had the highest radioactivity of any part of the corn plant. The radioactive grain contained a sufficient amount of radioactivity so that it proved satisfactory in a poultry feeding experiment.

The radioactive phosphorus was obtained from Oak Ridge through the assistance of Dr. E. C. Pollard of Yale University who also assisted in its preparation for safe handling.

Soils Department
Connecticut Agricultural Experiment Station
New Haven, Connecticut

NOTES

Purdue Section.—The Purdue section of the American Society of Plant Physiologists has concluded a very active and very profitable season marking the twenty-first since the Purdue section was founded. The 1947–48 program opened with summer meetings held July 24 and 25, 1947. On July 24, Dr. J. Fisher Stanfield, secretary for the National Society, opened the meeting with a discussion on "The American Institute of Biological Sciences as it pertains to the American Society of Plant Physiologists." Dr. E. J. Lund of the University of Texas was the guest speaker and presented three lectures and two round table discussions on "Bio-electrical Potentials and Growth Correlations." Eighty-six persons attended the dinner in his honor.

On October 20, 1947, the fall program opened with a dinner meeting in cooperation with the Sigma Xi and the American Biological Society. Dr. H. J. Muller, Geneticist and Professor of Zoology at the University of Indiana lectured on "The Production and Avoidance of Mutations." Dr. Muller was awarded the Nobel Prize in medicine and physiology for 1946.

On Nov. 3, Dr. J. E. Christian of the Department of Pharmacy at Purdue University lectured on "The Application of Radioactive Isotopes to Research and Medicine." Other Purdue speakers included Dr. A. A. Lindsay of the Biology Department who spoke on "Vegetation Types West of the Mississippi," Dr. John D. Hartman of the Department of Horticulture whose subject was "Experiences in Breeding Vegetable Crops at Purdue," Dr. A. J. Ullstrup, Department of Agricultural Botany, speaking on "Aversion Phenomena in Diplodia," Dr. H. E. Koffler, Department of Biology, his subject being "Biochemical Genetics," and Darrell McCloud of the Agronomy Department talking on "Nitrogen Relationships of Legume and Grass Mixtures."

On the spring program of 1948, the Purdue section was fortunate in having as their guest speaker, Dr. T. C. Broyer of the University of California. Dr. Broyer gave two lectures on March 16. His first lecture was entitled "Possible Metabolically Activated Mechanisms for the Movement of Water in Plants," and at a dinner meeting on the same day he talked on "Technic for the Study of Mineral Requirements of Plant and Observations for Microelement Deficiencies."

On February 17, Dr. E. W. Stark of the Department of Forestry at Purdue contributed to the program his talk on "Vessel Member and Fiber Length of Certain Native Woods." Other contributors from Purdue were Arthur Hobbs of the Department of Agronomy who lectured on "Boron Studies with Special Reference to Some Indiana Soils," Dr. O. E. Nelson of the Department of Agricultural Botany and Plant Pathology who chose as his topic, "Growth Correlates of Electromotive Forces in Maize Seeds," Robert Hoshaw of the Biology Department speaking on "Leaf Abscission

in the Genus Quercus," Dr. Laurenz Greene, Department of Horticulture, relating his experiences in "Horticulture Research in San Salvador," and Dr. J. Hugo Kraemer of the Department of Forestry who presented a discussion on "The Travels of a Forester in Northern Europe" at the annual spring dinner meeting on May 18, 1948, which 66 persons attended.

The chairman of the Purdue Section in 1947–48 was Dr. R. E. Girton and Dr. H. H. Kramer served as secretary-treasurer. The officers elected for the 1948–49 season are Dr. G. A. Gries of the Agricultural Botany Department as chairman and Dr. A. A. Lindsay of the Biology Department as secretary-treasurer.

Western Section.—Annual summer meetings of the Western Section, American Society of Plant Physiologists were held in Berkeley, California with the University of California as host institution. The society participated jointly in three symposia, all of which were well attended. One, sponsored by the plant physiologists, was entitled "Recent Developments in Plant Growth Regulators." The subjects included "Observations on Auxin Physiology," "Fruit Set and Growth Substances," "Growth Substances and Abscission," and "Auxins as Herbicides." The botanical and ecological societies sponsored a symposium on "Floristic and Ecological Aspects of the Great Basin and the South Western Deserts." The other, sponsored by the phytopathologists, was entitled "Biology of Fungi."

Eighteen papers were delivered by members at three half day sessions. These included a wide range of subjects of physiological, anatomical, and biochemical nature. Processes of growth, photosynthesis, and absorption and translocation of solutes were discussed; similarly, *in vivo* and *in vitro* studies on oxidation-reduction, enzymes, hormones, pigments and inorganic solutes. A joint banquet with members of the Botanical Society of America was enjoyed by about one hundred persons. A Biologists' dinner was also scheduled. The sessions were presided over by the incumbent and new elected chairmen of the Section viz., Dr. Biddulph, and Dr. Bonner. Professor Hoagland was a guest chairman. Officers for 1948–1949 are: J. Bonner, Chairman; T. C. Broyer, Vice chairman; H. B. Currier, Secretary-Treasurer.

New Directory.—It is necessary that all Directory cards be returned to the Executive Secretary-Treasurer if members desire any changes in data from that given in Bulletin 16. The Directory will go to press about November 1 and it should be in the mails soon after this date.

Chemical Composition of Plants as an Index of Their Nutritional Status.—D. W. Goodall and F. G. Gregory. Imperial Bureau of Horticulture and Plantation Crops, Sales Branch, Penglais; Aberystwyth, Wales. 167 pages, 1947. 9 shillings.

This publication appears as Technical Communication 17 of the Imperial Bureau of Horticulture and Plantation Crops. It is a critical study of

plant analysis including the minor elements as a means of determining the mineral requirements of plants. Recommendations for fertilizer treatments based on analytical data and the nutritional status of crops, particularly in early vegetative stages, are reviewed to determine validity of practical procedures.

The contents include sections on methods for the diagnoses of fertilizer requirements; early and recent research on diagnostic plant analysis; nutrient supply, uptake and internal concentration in relation to yield; analytical techniques; methods of expression and interpretation of data; factors affecting use of analyses for diagnosis and plant analysis in comparison with other methods of nutritional diagnosis. The authors have compiled the results of an enormous number of original works, expertly organized them into a condensed but very usable form. Several very extensive tables are alphabetized by crop for rapid reference. The Bibliography, alphabetized by author, contains 815 citations with complete titles. The separate subject and author indices are very comprehensive. The thoroughness of assembling research data in this field; the conciseness and clarity of its presentation and its critical evaluation by experts in pure and applied physiology will make this publication a standard reference work for many years.

Photosynthesis in Plants.—The second monograph of the Society has been announced for November publication. Twenty papers from the principal workers and laboratories interested in this basic process of plant physiology have been assembled under the leadership of Dr. James Franck of the University of Chicago. The topics have been planned to cover both the basic factors and the most recent advances in photosynthesis. Most of the papers read before the two-day symposium presented by Section C of the American Association for the Advancement of Science at Chicago are included. The discussions range from world totals of CO_2 fixation through photosynthesis under field conditions, the chemistry of photosynthetic products and plant pigments, to quantum efficiency and the most recent research on the intermediate products of CO_2 fixation. The book is planned for the plant scientist or chemist who wishes to bring himself abreast of the very large amount of intensive research on the topic. **Members of the Society** have the opportunity to obtain personal copies at a 25 per cent. discount from the list price of $6.00 by sending their checks for $4.50 to J. F. Stanfield, Miami University, Oxford, Ohio, before November 20.

GENERAL INDEX

A

Absorption and fluorescence spectra of two red marine algae, the 456

Acidity titratable, ascorbic acid, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr., diurnal changes and growth rates as associated with, 38

Action of tri-iodobenzoic acid on growth, the 158

Advances in Enzymology, Volume VII, edited by F. F. Nord, 264

Aerated potato discs, the role of active water absorption in auxin-induced water uptake by, 507

Agricultural Chemistry, an Introduction to, by N. M. Comber, H. T. Jones, and J. S. Willcox, 266

Algae, two red marine, the absorption and fluorescence spectra of, 455

Allison, F. E., Gaseous losses of nitrogen from green plants. I. Studies with Chlorella and Lemna, 496

II. Studies with excised leaves in nutrient media, 601

Altschul, A. M., A rapid method for the measurement of the inhibition of deterioration of intact seeds, 84

Ananas comosus (L.) Merr., diurnal changes and growth rates as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the leaves of, 38

Anderson, W. S., Effect of nutrient level on the growth and chemical composition of sweetpotatoes in sand cultures, 223

Andrews, F. R., A rapid method for the measurement of the inhibition of deterioration in intact seeds, 84

Andrews, W. B., The Response of Crops and Soils to Fertilizers and Manures, 166

Annual Review of Biochemistry, Volume XVI, 1947, edited by J. M. Luck, H. S. Long, and G. Mackinney, 264

Annual Review of Microbiology, Volume I, edited by C. E. Clifton, 400

Apple tissue, investigations on the fluorometric determination of malic and succinic acids in, 443

trees, young, the effect of varying amounts of potassium on the growth and potassium accumulation of, 112

Apples, pectic content of, in relation to thiocyanate sprays, 142

the determination of pectic materials in. The use of 19 per cent. hydrochloric acid and Pectinol A, 98

Architecture, Chemical, edited by R. E. Burk and O. Grummit, 265

Arnon, D. I., Plant and Soil, Volume I, 1948, edited by, 265

Arnoff, S., Phosphorus turnover and photosynthesis, 351

Ascorbic acid content of potato tubers, the influence of certain rootstocks and scions on the, 338

Attractants and Repellents, Chemical Insect, by Vincent G. Dethier, 399

Avena coleoptile to transversely applied direct current, electrical curvature responses of, 188

Avery, G. S., Jr., Hormones and Horticulture, 262

Avocados, the identification of ethylene as a volatile product of ripening, 526

B

Baeyens, J., Plant and Soil, Volume I, 1948, edited by, 265

Bailey, A. E., Cottonseed and Cottonseed Products: Their Chemistry and Technology, edited by, 264

Balance, cation-anion, in the leaves (II). Potassium deficiency and excess in guayule, 403

Barr, C. Guinn, Investigations on the fluorometric determination of malic and succinic acids in apple tissue, 443

Basic Botany, by Fred W. Emerson, 399

Bean stem tissue, the effect of 2,4-Dichlorophenoxyacetic acid on the respiratory metabolism of, 70

Best, A. H., Changes in chemical composition and biological activity of developing Tung fruit with reference to oil synthesis, 359

Biale, J. B., The identification of ethylene as a volatile product of ripening avocados, 526

BIBBEY, ROBERT O., Physiological studies of weed seed germination, 467
Bibliography of References to the Literature on the Minor Elements: fourth edition. Chilean Nitrate Education Bureau, 400
Biochemistry, Annual Review of, Volume XVI, 1947, edited by J. M. LUCK, H. S. LONG, G. MACKINNEY, 264
Biology, Plant, second edition, by P. WEATHERWAX, 263
Plant, The Essentials of, by F. D. KERN, 261
Blossoming and pigment content, 379
BONNER, W. D., JR., The action of tri-iodobenzoic acid on growth, 158
Boron and manganese on the growth and calcium uptake of blue lupine in solution culture. The effect of the interrelationship of, 609
Botanik der Gegenwart und Vorzeit, by K. F. W. JESSEN, 263
Botany, Basic, by FRED W. EMERSON, 399
BOUYOUCOS, G. J., A comparison of electric resistance units for making a continuous measurement of soil moisture under field conditions, 532
BROWN, ALLAN H., The carbohydrate constituents of scenedesmus in relation to the assimilation of carbon by photoreduction, 331
Bryophyllum calycinum, correction of data for protein nitrogen in leaves of, 149
the effect of temperature upon the culture of excised leaves of: studies in the metabolism of crassulacean plants, 123
BURK, R. E., Chemical Architecture, edited by, 265
BURSTROM, HANS, Corresponding membership, 256
Physiologica Plantarum, Volume I, edited by 398

C

Calcium, the effect of, and other ions on the early development of the radicle of cotton seedlings, 516
uptake of blue lupine in solution culture. The effect of the interrelationship of boron and manganese on the growth and, 609
CALVIN, M., Phosphorus turnover and photosynthesis, 351
Carbohydrate accumulation in the cotton plant at low moisture levels, 169
and nitrogenous fractions, ascorbic acid, and tritable acidity in the leaves of *Ananas comosus* (L.) Merr., diurnal changes and growth rates as associated with, 38
constituents of Scenedesmus in relation to the assimilation of carbon by photoreduction, the, 331
Carbon, the carbohydrate constituents of Scenedesmus in relation to the assimilation of, by photoreduction, 331
Carotene bodies to vitamin A, Contribution of studies with the electron microscope to studies of the relationship of chromoplasts to carotene bodies and, 621
Cation-anion balance in the leaves (II). Potassium deficiency and excess in guayule, 403
CHANDLER, ROBERT F., Forest Soils, 398
Changes in chemical composition and biological activity of developing tung fruit with reference to oil synthesis, 359
Chemical Architecture, edited by R. E. BURK and O. GRUMMIT, 265
Composition of Foods, the, by R. A. MCCANCE and E. M. WIDDOWSON, 266
Composition of Plants as an Index of Their Nutritional Status, by D. W. GOODALL and F. G. GREGORY, 640
Insect Attractants and Repellents, by VINCENT G. DETHIER, 399
Chemistry, Agricultural, an Introduction to, by N. M. COMBER, H. T. JONES, and J. S. WILLCOX, 266
and Technology, their—Cottonseed and Cottonseed Products, edited by A. E. BAILEY, 164
CHESTER, K. STARR, Nature and Prevention of Plant Diseases, 265
Chicago Meeting, American Society of Plant Physiologists, 165
Chilean Nitrate Education Bureau, Bibliography of References to the Literature on the Minor Elements: fourth edition, 400
Chlorella and Lemna, I. Studies with. Gaseous losses of nitrogen from green plants, 498
Chlorosis and nodulation of cowpeas as affected by trial sulphur applications

to calcareous soil in the greenhouse, 162
Chromoplasts to carotene bodies and carotene bodies to vitamin A, Contribution of studies with the electron microscope to studies of the relationship of, 621
CHUN, H. H. Q., Diurnal changes and growth rates as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr., 38
Cinchona ledgeriana, the effect of three factorial levels of nitrogen and phosphorus on the growth and composition of, 343
Cincinnati meeting, twenty-third annual, American Society of Plant Physiologists, 397
Citrus trees and fruit, effect of the rootstock on the composition of, 309
CLIFTON, C. E., Annual Review of Microbiology, Volume I, edited by, 400
COLLANDER, RUNAR, Corresponding membership, 255
COMBER, N. M., An Introduction to Agricultural Chemistry, 266
Comparison of electric resistance units for making a continous measurement of soil moisture under field conditions, a, 532
CONDON, M. Z., A rapid method for the measurement of the inhibition of deterioration in intact seeds, 84
COOIL, BRUCE J., Effects of potassium deficiency and excess upon certain carbohydrate and nitrogenous constituents in guayule, 425
Potassium deficiency and excess in guayule. I. Growth responses and mineral content, 286
Potassium deficiency and excess in guayule. II. Cation-anion balance in the leaves, 403
Corn and cotton seeds, relation of water to the germination of, 201
(maize), high-Niacin, a survey of the possibilities and methods of breeding, 238
mineral accumulation in, 267
Correction of data for protein nitrogen in leaves of *Bryophyllum calycinum*, 149
Corresponding members, HANS BURSTROM, 256
RUNAR COLLANDER, 255
ALBERT FREY-WYSSLING, 256
F. G. GREGORY, 255
Cotton plant, carbohydrate accumulation in the, at low moisture levels, 169
seedlings, the effect of calcium and other ions on the early development of the radicle of, 516
seeds, relation of water to the germination of corn and, 201
Cottonseed and Cottonseed Products: Their Chemistry and Technology, edited by A. E. BAILEY, 264
Cowpeas, chlorosis and nodulation of, as affected by trial sulphur applications to calcarious soil in the greenhouse, 162
Crassulacean plants, studies in the metabolism of: the effect of temperature upon the culture of excised leaves of *Bryophyllum calycinum*, 123
CROCKER, W., growth of plants, 262
Crops and Soils, the Response of, to Fertilizers and Manures, by W. B. ANDREWS, 166
Diseases of Field, by JAMES G. DICKSON, 399
CRUZADO, H. J., Note on flower formation in the pineapple induced by low night temperatures, 282
Cut shoots after wilting, recovery of turgor by, 152

D

DAUBENMIRE, R. F., Plants and Environment, 263
DAWSON, RAY F., A survey of the possibilities and methods of breeding high-niacin corn (maize), 238
Deterioration, inhibition of, in intact seeds, a rapid method for the measurement of the, 84
Determination of pectic materials in apples, the. The use of 19 per cent. hydrochloric acid and Pectinol A, 98
DETHIER, VINCENT G., Chemical Insect Attractants and Repellents, 399
DICKSON, JAMES G., Dieseases of Field Crops, 399
Diseases of Field Crops by JAMES G. DICKSON, 399

Diurnal changes and growth rates as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr., 38
fluctuations of growth in length of tomato stem, 156
DROSDOFF, M., Changes in chemical composition and biological activity of developing tung fruit with reference to oil synthesis, 359
DUSTMAN, R. B., Pectic content of apples in relation to thiocyanate sprays, 142
The determination of pectic materials in apples. The use of 19 per cent. hydrochloric acid and Pectinol A, 98

E

EATON, FRANK M., Carbohydrate accumulation in the cotton plant at low moisture levels, 169
EDGERTON, L. J., The effect of varying amounts of potassium on the growth and potassium accumulation of young apple trees, 112
Effect of calcium and other ions on the early development of the radicle of cotton seedlings, the, 516
of 2,4-dichlorophenoxyacetic acid on the respiratory metabolism of bean stem tissue, the 70
of nutrient level on the growth and chemical composition of sweetpotatoes in sand cultures, 223
of some environmental factors on the movements of the guard cells, the, 5
of the rootstock on the composition of citrus trees and fruit, 309
of temperature upon the culture of excised leaves of *Bryophyllum calycinum*, the: studies in the metabolism of crassulacean plants, 123
of three factorial levels of nitrogen and phosphorus on the growth and composition of *Cinchona ledgeriana*, the 343
of varying amounts of potassium on the growth and potassium accumulation of young apple trees, the 112
Effects of potassium deficiency and excess upon certain carbohydrate and nitrogenous constituents in guayule, 425
Election of officers, 1948–49, American Society of Plant Physiologists, 397
Electric resistence units for making a continuous measurement of soil moisture under field conditions, a comparison of, 532
Electrical and curvature responses of the Avena coleoptile to transversely applied direct current, 188
Elements, Bibliography of References to the Literature on the Minor: fourth edition. Chilean Nitrate Education Bureau, 400
EMERSON, FRED W., Basic Botany, 399
Environment, Plants and, by R. F. DAUBENMIRE, 263
Environmental factors on the movements of the guard cells, the effect of some, 5
Enzymology, Advances in, Volume VII, edited by F. F. NORD, 264
ERGLE, D. R., Carbohydrate accumulation in the cotton plant at low moisture levels, 169
Essentials of Plant Biology, the, by F. D. KERN, 261
Ethylene, the identification of, as a volatile product of ripening avocados, 526

F

Factor method for the area of tomato leaves, a, 634
Fertilizers and Manures, the Response of Crops and Soils to, by W. B. ANDREWS, 166
FEVOLD, H. L., Vitamin C content of walnuts (Persian) during growth and development, 133
Field Crops, Diseases of, by JAMES G. DICKSON, 399
FISH, V. B. Pectic content of apples in relation to thiocyanate sprays, 142
The determination of pectic materials in apples. The use of 19 per cent. hydrochloric acid and Pectinol A, 98
Flower formation in the pineapple induced by low night temperatures, note on, 282
Fluctuations, diurnal, of growth in length of tomato stem, 156
Fluorescence spectra of two red marine algae, the absorption and, 455
Fluorometric determination of malic and succinic acids in apple tissue, investigations on the, 443

Foods, the Chemical Composition of, by R. A. McCance and E. M. Widdowson, 266
Forest Soils, by Harold J. Lutz, and Robert F. Chandler, 398
Freeland, R. O., Photosynthesis in relation to stomatal frequency and distribution, 595
French, C. S., The absorption and fluorescence spectra of two red marine algae, 455
Frenching of tobacco, Soil temperature as a factor in the, 560
Frenching of tobacco, Unavailability of iron as a cause of, 576
Frey-Wyssling, Albert, Corresponding membership, 256
Fulbright Act, the 260

G

Gaddy, V. L., Gaseous losses of nitrogen from green plants. I. Studies with Chlorella and Lemna, 496
Gaseous losses of nitrogen from green plants. I. Studies with Chlorella and Lemna, 496
II. Studies of excised leaves in nutrient media, 601
Gauch, H. G., Rate of leaf elongation as affected by the intensity of the total soil moisture stress, 485
Gegenwart und Vorzeit, Botanik der, by K. F. W. Jessen, 263
Germination of corn and cotton seeds, relation of water to the, 201
weed seed, physiological studies of, 467
Gerretsen, F. C., Plant and Soil, Volume I, 1948, edited by, 265
Gieger, Marvin, Effect of nutrient level on the growth and chemical composition of sweetpotatoes in sand cultures, 223
Ginter, W. D., Correction of data for protein nitrogen in leaves of *Bryophyllum calycinum*, 149
Studies in the metabolism of crassulacean plants: the effect of temperature upon the culture of excised leaves of *Bryophyllum calycinum*, 123
Goodall, D. W., Chemical Composition of Plants as an Index of Their Nutritional Status, 640
Graham, Edward H., The Land and Wildlife, 266
Grass, Two Blades of, by T. Swann Harding, 400
Green plants, gaseous losses of nitrogen from. I. Studies with Chlorella and Lemna, 496
Gregory, F. G., Chemical Composition of Plants as an Index of Their Nutritional Status, 640
Corresponding membership, 255
Growth and chemical composition of sweet potatoes in sand cultures, effect of nutrient level on the, 223
and potassium accumulation of young trees, the effect of varying amounts of potassium on the, 112
of Plants, by W. Crocker, 262
rates and diurnal changes as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr., 38
responses and mineral content. I. Potassium deficiency and excess in guayule, 286
the action of tri-iodobenzoic acid on, 158
Grummit, O., Chemical Architecture, edited by, 265
Guard cells, the effect of some environmental factors on the movements of the, 5
Guayule, effects of potassium deficiency and excess upon certain carbohydrate and nitrogenous constituents in, 425
potassium deficiency and excess in. I. Growth responses and mineral content, 286
potassium deficiency and excess in. II. Cation-anion balance in the leaves, 403
Gustafson, Felix G., Influence of light intensity upon the concentration of thiamin and riboflavin in plants, 373

H

Haas, A. R. C., Effect of the rootstock on the composition of citrus trees and fruit, 309
Harding, T. Swann, Two Blades of Grass, 400
Henderson, J. H. M., The effect of the interrelationship of boron and manganese on the growth and calcium uptake of blue lupine in solution culture, 609

Hormones and Horticulture, by G. S. AVERY, JR., and E. B. JOHNSON, 262
Hydrochloric acid and Pectinol A, the use of 19 per cent. The determination of pectic materials in apples, 98

I

Identification of ethylene as a volatile product of ripening avocados, The, 526
Influence of certain rootstocks and scions on the ascorbic acid content of potato tubers, the 338
of light intensity upon the concentration of thiamin and riboflavin in plants, 373
Inhibition of deterioration in intact seeds, a rapid method for the measurement of the, 84
Insect Attractions and Repellents, Chemical, by VINCENT G. DETHIER, 399
Intact seeds, a rapid method for the measurement of the inhibition of deterioration in, 84
Intensity of the total soil moisture stress, rate of leaf elongation as affected by the, 485
Introduction to Agricultural Chemistry, an, by N. M. COMBER, H. T. JONES, and J. S. WILLCOX, 266
Investigations on the fluorometric determination of malic and succinic acids in apple tissue, 443
Iron, unavailability of, as a cause of frenching in tobacco, 576
IVANOFF, S. S., Chlorosis and nodulation of cowpeas as affected by trial sulphur applications to calcareous soil in the greenhouse, 162

J

JACOBSON, H. G. M., A technique for introducing radioactive phosphorus into the grain of growing corn, 636
JENSEN, E., A rapid method for the measurement of the inhibition of deterioration in intact seeds, 84
JESSEN, K. F. W., Botanik der Gegenwart und Vorzeit, 263
JOHNSON, E. B., Hormones and Horticulture, 262
JOHNSON, EDNA L., Response of *Kalanchoe tubiflora* to x-radiation, 544
JOHNSTON, EARL STEINFORD, obit., 256
JONES, H. T., An Introduction to Agricultural Chemistry, 266
JONES, L. H., Soil temperature as a factor in the frenching of tobacco, 560
Unavailability of iron as a cause of frenching in tobacco, 576
JOST, LUDWIG, obit., 259

K

Kalanchoe tubiflora, response of, to x-radiation, 544
KELLY, W. C., The influence of certain rootstocks and scions on the ascorbic acid content of potato tubers, 338
KERN, F. D., The Essentials of Plant Biology, 261
KLOSE, A. A., Vitamin C content of walnuts (Persian) during growth and development, 133

L

LAMBOU, M. G., A rapid method for the measurement of the inhibition of deterioration in intact seeds, 84
Land and Wildlife, the, by E. H. GRAHAM, 266
Leaf elongation as affected by the intensity of the total soil moisture stress, rate of, 485
LEAVENWORTH, C. S., Correction of data for protein nitrogen in leaves of *Bryophyllum calycinum*, 149
Studies in the metabolism of crassulacean plants: the effect of temperature upon the culture of excised leaves of *Bryophyllum calycinum*, 123
Lemna and Chlorella, I. Studies with. Gaseous losses of nitrogen from green plants, 496
LEONARD, O. A., Effect of nutrient level on the growth and chemical composition of sweetpotatoes in sand cultures, 223
The effect of calcium and other ions on the early development of the radicle of cotton seedlings, 516
LEVITT, J., The role of active water absorption in auxin-induced water uptake by aerated potato discs, 505
Light intensity, influence of, upon the concentration of thiamin and riboflavin in plants, 373

Literature on the Minor Elements, Bibliography of References to: fourth edition. Chilean Nitrate Education Bureau, 400

LIVINGSTON, BURTON EDWARD, In memoriam, iii

LLOYD, FRANCES ERNEST, In memoriam, 1

LONG, H. S., Annual Review of Biochemistry, Volume XVI, 1947, edited by, 264

LOUSTALOT, A. J., The effect of three factorial levels of nitrogen and phosphorus on the growth and composition of *Cinchona ledgeriana*, 343

LOVE, KATHARINE S., Gaseous losses of nitrogen from green plants. I. Studies with Chlorella and Lemna, 496

LUCK, J. M., Annual Review of Biochemistry, Volume XVI, 1947, edited by, 264

Lupine in solution culture, The effect of the interrelationship of boron and manganese on the growth and calcium uptake of blue, 609

LUTZ, HAROLD J., Forest Soils, p. 398

LYON, CHARLES J., A factor method for the area of tomato leaves, 634

M

MACDOWALL, F. D. H., The absorption and fluorescence spectra of two red marine algae, 455

MACKINNEY, G., Annual Review of Biochemsitry, Volume XVI, 1947, edited by, 264

Malic and succinic acids in apple tissue, investigations on the fluorometric determinations of, 443

Manganese on the growth and calcium uptake of blue lupine in solution culture, The effect of the interrelationship of boron and, 609

Manures and Fertilizers, the Response of Crops and Soils to, by W. B. ANDREWS, 166

MCCANCE, R. A., The Chemical Composition of Foods, 266

McIntosh apple leaves, a microchemical examination of, showing relationship of cell wall constituents to penetration of spray solutions, 557

MEADE, R. C., Pectic content of apples in relation to thiocyanate sprays, 142

The determination of pectic materials in apples. The use of 19 per cent. hydrochloric acid and Pectinol A, 98

Measurement of soil moisture under field conditions, a comparison of electric resistence units for making a continuous, 532

of the inhibition of deterioration in intact seeds, a rapid method for the, 84

Meeting, Chicago, American Society of Plant Physiologists, 165

Cincinnati, twenty-third annual, American Society of Plant Physiologists 397

Metabolism of crassulacean plants, studies in the: the effect of temperature upon the culture of excised leaves of *Bryophyllum calycinum*, 123

Method, a rapid, for the measurement of the inhibition of deterioration in intact seeds, 84

Methods of breeding high-niacin corn (maize). A survey of the possibilities and, 238

MICK, A. H., A comparison of electric resistance units for making a continuous measurement of soil moisture under field conditions, 532

Microbiology, Annual Review of, Volume I, edited by C. E. CLIFTON, 400

Microchemical examination of McIntosh apple leaves showing relationship of cell wall constituents to penetration of spray solutions, a, 557

Mineral accumulation in corn, 267

content, I. Growth responses and. Potassium deficiency and excess in guayule, 286

Minor Elements, Bibliography of References to the Literature on the: fourth edition. Chilean Nitrate Education Bureau, 400

Movements of the guard cells, the effect of some environmental factors on the, 5

MULDER, E. G., Plant and Soil, Volume I, 1948, edited by, 265

N

Nature and Prevention of Plant Diseaees by K. STARR CHESTER, 265

Nitrogen, gaseous losses of, from green plants. I. Studies with Chlorella and Lemna, 496

II. Studies with excised leaves in nutrient media, 601

the effect of three factorial levels of, and phosphorus on the growth and composition of *Cinchona ledgeriana*, 343
Nitrogenous and carbohydrate fractions, ascorbic acid, and titratable acidity in the leaves of *Ananas comosus* (L.) Merr., diurnal changes and growth rates as associated with, 38
Nodulation and chlorosis of cowpeas as affected by trial sulphur applications to calcareous soil in the greenhouse, 162
NORD, F. F., Advances in Enzymology, Volume VII, edited by, 264
Note on flower formation in the pineapple induced by low night temperatures, 282
Nuclear division, protoplasmic changes during, in pollen mother-cells and microspores of *Trillium erectum*, 388
Nutritional Status, Chemical Composition of Plants as an Index of Their, by D. W. GOODALL and F. G. GREGORY, 640

O

Oil synthesis, changes in chemical composition and biological activity of developing tung fruit with reference to, 359
OOSTING, HENRY J., The Study of Plant Communities, 401

P

PALMITER, D. H., A microchemical examination of McIntosh apple leaves showing relationship of cell wall constituents to penetration of spray solutions, 557
PEAT, JEAN, Vitamin C content of walnuts (Persian) during growth and development, 133
Pectic content of apples in relation to thiocyanate sprays, 142
materials in apples, the determination of. The use of 19 per cent. hydrochloric acid and Pectinol A, 98
Pectinol A and hydrochloric acid, the use of 19 per cent. The determination of pectic materials in apples, 98
Phosphorus, the effect of three factorial levels of nitrogen and, on the growth and composition of *Cinchona ledgeriana*, 343
turnover and photosynthesis, 351
into grain of growing corn. A technique for introducing radioactive, 636
Photoreduction, the carbohydrate constituents of Scenedesmus in relation to the assimilation of carbon by, 331
Photosynthesis, phosphorus turnover and, 351
in relation to stomatal frequency and distribution, 595
Physiologica Plantarum, Volume I, edited by HANS BURSTROM, 398
Physiological studies of weed seed germination, 467
Pigment content, blossoming and, 379
PINCK, L. A., Gaseous losses of nitrogen from green plants. I. Studies with Chlorella and Lemna, 496
Pineapple, note on flower formation in the, induced by low night temperatures, 282
Plant and Soil, Volume I, 1948, edited by D. I. ARNON, J. BAEYENS, F. C. GERRETSEN, E. G. MULDER, and A. C. SCHUFFELEN, 265
Biology, second edition, by P. WEATHERWAX, 263
Biology, the Essentials of, by F. D. KERN, 261
Communities, the Study of, by HENRY J. OOSTING, 401
Diseases, Nature and Prevention of, by K. STARR CHESTER, 265
Plantarum, Physiologica, by HANS BURSTROM, Volume I, 398
Plants and Environment by R. F. DAUBENMIRE, 263
Chemical Composition of, as an Index of Their Nutritional Status, by D. W. GOODALL and F. G. GREGORY, 640
growth of, by W. CROCKER, 262
Potassium deficiency and excess, effects of, upon certain carbohydrate and nitrogenous constituents in guayule, 426
deficiency and excess in guayule. I. Growth responses and mineral content, 286
deficiency and excess in guayule. II. Cation-anion balance in the leaves, 403
the effect of varying amounts of, on the growth and potassium accumulation of young apple trees, 112

Potato tubers, the influence of certain rootstocks and scions on the ascorbic acid content of, 338
PRATT, H. K., The identification of ethylene as a volatile product of ripening avocados, 526
PRESLEY, J. T., The effect of calcium and other ions on the early development of the radicle of cotton seedlings, 516
Prevention, Nature and, of Plant Diseases by K. STARR CHESTER, 265
Protein nitrogen in leaves of *Bryophyllum calycinum*, correction of data for, 149
Protoplasmic changes during nuclear division in pollen mother-cells and microspores of *Tillium erectum*, 388
PRYANNISHNIKOV, DMITRIJ N., obit., 397
PUCHER, GEORGE WALTER, obit., 258
Correction of data for protein nitrogen in leaves of *Bryophyllum calycinum*, 149
Studies in the metabolism of crassulacean plants: the effect of temperature upon the culture of excised leaves of *Bryophyllum calycinum*, 123

R

Radioactive phosphorus into the grain of growing corn. A technique for introducing, 636
Rapid method for the measurement of the inhibition of deterioration in intact seeds, a, 84
Rate of leaf elongation as affected by the intensity of the total soil moisture stress, 485
RAYNER, M. C., Trees and Toadstools, 265
Recovery of turgor by cut shoots after wilting, 152
References to the Literature on Minor Elements, Bibliography of: fourth edition. Chilean Nitrate Education Bureau, 400
Relation of water to the germination of corn and cotton seeds, 201
Repellents, Chemical Insect Attractants and, by VINCENT G. DETHIER, 399
Resistance units (electric), for making a continuous measurement of soil moisture under field conditions, a comparison of, 532
Respiratory metabolism of bean stem tissue, the effect of 2,4-dichlorophenoxyacetic acid on the, 70
Response of Crops and Soils to Fertilizers and Manures, the, by W. B. ANDREWS, 166
of *Kalanchoe tubiflora* to x-radiation, 544
REUTHER, WALTER, Changes in chemical composition and biological activity of developing tung fruit with reference to oil synthesis, 359
Review of Microbiology, Annual, by C. E. CLIFTON, 400
Riboflavin in plants, influence of light intensity upon the concentration of thiamin and, 373
RICHEY, F. D., A survey of the possibilities and methods of breeding high-niacin corn (maize), 238
ROBERTS, E. A., A microchemical examination of McIntosh apple leaves showing relationship of cell wall constituents to penetration of spray solutions, 557
Contribution of studies with the electron microscope to studies of the relationship of chromoplasts to carotene bodies and bodies to vitamin A, 621.
ROBERTS, R. H., Blossoming and pigment content, 379
Role of active water absorption in auxin-induced water uptake by aerated potato discs, the, 505
Rootstock, effect of the, on the composition of citrus trees and fruit, 309
Rootstocks and scions, the influence of certain, on the ascorbic acid content of potato tubers, 338

S

SAYRE, J. D., Mineral accumulation in corn, 267
Scenedesmus, the carbohydrate constituent of, in relation to the assimilation of carbon by photoreduction, 331
SCHRANK, A. R., Electrical and curvature responses of the Avena coleoptile to transversely applied direct current, 188
SCHUFFELEN, A. C., Plant and Soil, Volume I, 1948, edited by, 265
Scions, the influence of certain rootstocks and, on the ascorbic acid content of potato tubers, 338

SELL, H. M., Changes in chemical composition and biological activity of developing tung fruit with reference to oil synthesis, 359

SIDERIS, C. P., Diurnal changes and growth rates as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr., 38

SLATTERY, M. C., Effects of potassium deficiency and excess upon certain carbohydrate and nitrogenous constituents in guayule, 425

SMITH, F. G., The effect of 2,4-dichlorophenoxyacetic acid on the respiratory metabolism of bean stem tissue, 70

Soil moisture stress, rate of leaf elongation as affected by the intensity of the total, 485

moisture under field conditions, a comparison of electric resistance units for making a continuous measurement of, 532

Plant and, Volume I, 1948, edited by D. I. ARNON, J. BAEYENS, F. C. GERRETSEN, E. G. MULDER, and A. C. SCHUFFELEN, 265

Soils and Crops, the Response of, to Fertilizers and Manures, by W. B. ANDREWS, 166

Forest, by HAROLD J. LUTZ and ROBERT F. CHANDLER, 398

SOMERS, G. FRED, The influence of certain rootstocks and scions on the ascorbic acid content of potato tubers, 338

SOUTHWICK, M. D., A microchemical examination of McIntosh apple leaves showing relationship of cell wall constituents to penetration of spray solutions, 557

Contribution of studies with the electron microscope to studies of the relationship of chromoplasts to carotene bodies and carotene bodies to vitamin A, 621

Spray solutions, a microchemical examination of McIntosh apple leaves showing relationship of cell wall constituents to penetration of, 557

STERLING, L. D., Gaseous losses of nitrogen from green plants. II. Studies with excised leaves in nutrient media, 601

STERN, HERBERT, Protoplasmic changes during nuclear division in pollen mother-cells and microspores of *Trillium erectum*, 388

STILES, I. E., Relation of water to the germination of corn and cotton seeds, 201

STOCKING, C. R., Recovery of turgor by cut shoots after wilting, 152

Stomatal frequency and distribution, Photosynthesis in relation to, 595

Studies in the metabolism of crassulacean plants: the effect of temperature upon the culture of excised leaves of *Bryophyllum calycinum*, 123

with Chlorella and Lemna (I). Gaseous losses of nitrogen from green plants, 496

Study of Plant Communities, the, by HENRY J. OOSTING, 401

Succinic acids in apple tissue, investigations on the fluorometric determination of malic and, 443

Sulphur applications, trial, to calcareous soil in the greenhouse, chlorosis and nodulation of cowpeas as affected by, 162

Survey of the possibilities and methods of breeding high-niacin corn (maize), a, 238

Sweetpotatoes in sand cultures, effect of the nutrient level on the growth and chemical composition of, 223

T

2,4-dichlorophenoxyacetate acid on the respiratory metabolism of bean stem tissue, the effect of, 70

TAYLOR, DAVID LAWRENCE, obit., 259

Technology and Chemistry, Their—Cottonseed and Cottonseed Products, edited by A. E. BAILEY, 264

Temperature, the effect of, upon the culture of excised leaves of *Bryophyllum calycinum:* Studies in the metabolism of crassulacean plants, 123

soil, as a factor in the frenching of tobacco, 560

Temperatures, note on flower formation in the pineapple induced by low night, 282

Thiamin and riboflavin in plants, influence of light intensity upon the concentration of, 373

THIMANN, K. V., The action of tri-iodobenzoic acid on growth, 158

Thiocyanate sprays, pectic content of apples in relation to, 142
TIO, M. A., Unavailability of iron as a cause of frenching of tobacco, 576
Titratable acidity, ascorbic acid, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr., diurnal changes and growth rates as associated with, 38
Toadstools, Trees and, by M. C. RAYNER, 265
Tobacco, soil temperature as a factor in the frenching of, 560
Unavailability of iron as a cause of frenching of, 576
Tomato leaves, a factor method for the area of, 634
stem, diurnal fluctuations of growth in length of, 156
Trees and Toadstools, by M. C. RAYNER, 265
Tri-iodobenzoic acid on growth, the action of, 158
Trillium erectum, protoplasmic changes during nuclear division in pollen mother-cells and microspores of, 338
Tung fruit, changes in chemical composition and biological activity of developing, with reference to oil synthesis, 359
Turgor, recovery of, by cut shoots after wilting, 152
Two Blades of Grass, by T. SWANN HARDING, 400

U

Use of 19 per cent. hydrochloric acid and Pectinol A, the. The determination of pectic materials in apples, 98

V

VAN NORMAN, R. W., The absorption and fluorescence spectra of two red marine algae, 455
VAN OVERBEEK, J., Note on flower formation in the pineapple induced by low night temperatures, 282
VEAL, M. P.,The effect of the interrelationship of boron and manganese on the growth and calcium uptake of blue lupine in solution culture, 609
VICKERY, H. B., Correction of data for protein nitrogen in leaves of *Bryophyllum calycinum*, 149
Studies in the metabolism of crassulacean plants: the effect of temperature upon the culture of excised leaves of *Bryophyllum calycinum*, 123
Vitamin C content of walnuts (Persian) during growth and development, 133
Vorzeit, Gegenwart und, Botanik der, by K. F. W. JESSEN, 263

W

WADLEIGH, C. H., Rate of leaf elongation as affected by the intensity of the total soil moisture stress, 485
Walnuts (Persian), vitamin C content of, during growth and development, 133
Water absorption in auxin-induced water uptake by aerated potato discs, the role of active, 505
relation of, to the germination of corn and cotton seeds, 201
WEATHERWAX, P., Plant Biology, second edition, 263
Weed seed germination, physiological studies of, 469
WIDDOWSON, E. M., The Chemical Composition of Foods, 266
Wildlife, the Land and, by E. H. GRAHAM, 266
WILLCOX, J. S., An Introduction to Agricultural Chemistry, 266
WILSON, C. C., Diurnal fluctuations of growth in length of tomato stem, 156
The effect of some environmental factors on the movements of the guard cells, 5
Wilting, recovery of turgot by cut shoots after, 152
WINTERS, HAROLD F., The effect of three factorial levels of nitrogen and phosphorus on the growth and composition of *Cinchona ledgeriana*, 343

X

X-radiation, response of *Kalanchoe tubiflora* to, 544

Y

YOUNG, H. Y., Diurnal changes and growth rates as associated with ascorbic acid, titratable acidity, carbohydrate and nitrogenous fractions in the leaves of *Ananas comosus* (L.) Merr., 38
YOUNG, R. E., The identification of ethylene as a volatile product of ripening avocados, 526

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CONTENTS—(*Continued*)

The Role of Actied Water Absorption in Auxin-Induced Water Uptake by Aerated Potato Discs. J. LEVITT, University of Missouri, Columbia 505

The Effect of Calcium and Other Ions on the Early Development of the Radicle of Cotton Seedlings. J. T. PRESLEY and O. A. LEONARD, Mississippi Agricultural Experiment Station, State College, Mississippi 516

The Identification of Ethylene as a Volatile Product of Ripening Avocados. H. K. PRATT, R. E. YOUNG, and J. B. BIALE, Division of Truck Crops, University of California, Davis, and Division of Subtropical Horticulture, Los Angeles 24 526

A Comparison of Electric Resistance Units for Making a Continuous Measurement of Soil Moisture Under Field Conditions. G. J. BOUYOUCOS and A. H. MICK, Michigan State College, East Lansing 532

Response of Kalanchoe tubiflora to X-Radiation. EDNA L. JOHNSON University of Colorado, Boulder 544

A Microchemical Examination of McIntosh Apple Leaves Showing Relationship of Cell Wall Constituents to Penetration of Spray Solutions. E. A. ROBERTS, M. D. SOUTHWICK, and D. H. PALMITER, Vassar College, Poughkeepsie, New York and New York State Agricultural Experiment Station, Geneva 557

Soil Temperture as a Factor in the Frenching of Tobacco (Nicotiana Tabacum L.). LINUS H. JONES, Massachusetts Agricultural Experiment Station, Amherst 560

Unavailability of Iron as a Cause of Frenching of Tobacco (Nicotiana Tabacum L.). LINUS H. JONES and MARCO A. TIO, Massachusetts Agricultural Experiment Station, Amherst 576

Photosynthesis in Relation to Stomatal Frequency and Distribution. R. O. FREELAND, Northwestern University, Evanston, Illinois 595

Gaseous Losses of Nitrogen from Green Plants. II. Studies with Excised Leaves in Nutrient Media. F. E. ALLISON and LUANN DETAR STERLING, U. S. Department of Agriculture, Beltsville 601

The Effect of the Interrelationship of Boron and Manganese on the Growth and Calcium Uptake of Blue Lupine (Lupinus angustifolius L.) in Solution Culture. JAMES H. HENDERSON and MARY P. VEAL, Tuskegee Institute, Alabama 609

Contributions of Studies with the Electron Microscope to Studies of the Relationship of Chromoplasts to Carotene Bodies and Carotene Bodies to Vitamin A. EDITH A. ROBERTS and MILDRED D. SOUTHWICK, Vassar College, Poughkeepsie, New York 621

Brief Papers

A Factor Method for the Area of Tomato Leaves. CHARLES J. LYON, Dartmouth College, Hanover, New Hampshire 634

A Technique for Introducing Radioactive Phosphorus into the Grain of Growing Corn. H. G. M. JACOBSON, Agricultural Experiment Station, New Haven, Connecticut 636

Notes 638

General Index, Volume 23 641